The use of stem cells and minimally invasive implantation techniques for the development of clinically relevant heart valve tissue engineering concepts

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The Use of Stem Cells and Minimally Invasive Implantation Techniques for the Development of Clinically relevant Heart Valve Tissue Engineering Concepts

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Foreword

The studies summarized in this habilitation thesis were performed at the Division of Surgical Research, University and University Hospital Zurich (Scientific Director: Prof. Dr. med. Dr. rer nat. Simon P. Hoerstrup) and at the Clinic for Cardiovascular Surgery (Director: Prof. Dr. med. Volkmar Falk), both University and University Hospital Zurich. The habilitation thesis is based on a selection of five publications which can be summarized under the following heading: `***The Use of Stem Cells and Minimally Invasive Implantation Techniques for the Development of Clinically relevant Heart Valve Tissue Engineering Concepts***`

The following publications are presented and attached in the appendix:

1. Schmidt D, Dijkman PE, Driessen-Mol A, Stenger R, Mariani C, Puolakka A, Rissanen M, Deichmann T, Odermatt B, Weber B, **Emmert MY**, Zund G, Baaijens FP, Hoerstrup SP. *Minimally-invasive implantation of living tissue engineered heart valves: a comprehensive approach from autologous vascular cells to stem cells.* J Am Coll Cardiol. 2010 Aug 3;56(6):510-20.

2. Cummings I, George S, Kelm J, Schmidt D, **Emmert MY**, Weber B, Falk V, Zünd G, Hoerstru*p* SP. *Tissue engineered vascular graft remodeling in a growing lamb model: Expression of Matrix Metalloproteinases*. Eur J Cardiothorac Surg. 2011 Apr 27. [Epub ahead of print].

3. **Emmert MY*,** Weber B*, Scherman J*, Gruenenfelder J, Verbeek R, Bracher M, Black M, Kortsmit J, Franz T, Schoenauer R, Baumgartner L, Brokopp C, Agarkova I, Wolint P, Zund G, Falk Zilla P, Hoerstrup SP. *Injectable Living Marrow Stromal Cell-based Autologous Tissue Engineered Heart Valves – First Experiences with a One-Step Intervention in Primates.* Eur Heart J. 2011 Mar 17. [Epub ahead of print]; *contributed equally

4. **Emmert MY,** Weber B, Behr L, Frauenfelder T, Brokopp CE, Grunenfelder J, Falk V, Hoerstrup SP. *Transapical Aortic Implantation of Autologous Marrow Stromal Cell-based Tissue Engineered Heart Valves – First Experiences in the Systemic Circulation.* JACC Cardiovasc Interv. 2011 Jul;4(7):822-3.

5. **Emmert MY*,** Weber B*, Behr L, Brokopp CE, Frauenfelder T, Kretschmar O, Falk V, Hoerstrup SP. *Fetal Transapical Stent Delivery into the Pulmonary Artery: Prospects for prenatal heart valve implantation.* Eur J Cardiothorac Surg. 2011 Jul 7. [Epub ahead of print]; *contributed equally

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1. Introduction

Valvular heart disease represents a major cause of morbidity and mortality worldwide (1, 2). Besides conventional therapy concepts based on surgical repair or replacement, transcatheter valve implantation techniques represent a rapidly evolving alternative for the treatment of such patients. The increasing implementation of these techniques into the clinical routine may have a major impact on the treatment strategy of patients with valvular heart disease (3). Various percutaneous catheter-based as well as transapical surgical implantation approaches have been developed and were successfully applied in both settings, experimental as well as in the clinical arena, representing a promising alternative to conventional heart valve surgery (4-6). Despite these significant advances, the currently available valvular constructs for minimally invasive, transcatheter-based procedures are bio-prosthetic and as such inherently prone to progressive degeneration and calcification suggesting their clinical indication primarily in the elderly, high-risk patients (7).

Tissue engineering approaches aiming at the fabrication of cell based, living heart valves have created substantial clinical hope and expectations to overcome these limitations. Different concepts using various cell sources and scaffold materials have been introduced over the past decade. The major aim of tissue engineering technologies is to generate tissue constructs based on their cellular components which comprise the characteristics of their native counterpart. In addition to long-term durability and sufficient mechanical properties, the absence of immunogenic or inflammatory responses represents a key factor for a functional tissue engineered heart valve. This aim can be achieved with a completely autologous tissue engineering concept.

The success of this approach depends on three key aspects: (1) a biodegradable and biocompatible matrix (scaffold) which determines the three-dimensional shape and serves as an initial guiding structure for cell attachment and tissue development; (2) an easy accessible

cell source from which a native-analogous living tissue can be generated; and (3) either sophisticated in vitro culture conditions which enable adequate neo-tissue formation resulting in implantable, living, autologous heart valve constructs, or in vivo concepts using directly implantable matrices with potential of in vivo remodeling and cell in-growth.

Particularly for pediatric applications, the growth potential of tissue engineered heart valves addresses an unmet medical and may prevent children with congenital malformations from high risk redo operations. Therefore, this concept may extend the future clinical application of trans-catheter valves beyond elderly high-risk patients to a broader patient population, including young patients (8). Furthermore the combination with stem cells may further increase the regenerative potential of tissue-engineered constructs. In particular, marrow stromal derived stem cells have been repeatedly suggested to represent an ideal cell source for cardiovascular tissue engineering. A clinically relevant heart valve tissue engineering concept would ideally comprise minimally-invasive techniques for both, cell harvest and valve implantation.

A brief overview about current *state-of-the-art* treatment strategies for valvular heart disease and its current limitation along with the principle concept of clinically relevant heart valve tissue engineering is provided.

1.1. Valvular Heart Disease

Valvular heart disease represents a major global disease load with an increasing number of patients in the developed world suffering from degenerative valve disease as well as an increasing patient cohort in the developing countries due to rheumatic diseases (1, 2, 9). For this reason it remains to be a significant cause of morbidity and mortality worldwide (10). More than 250,000 heart valve replacements are performed worldwide per year with a continuously increasing tendency that is expected to have tripled by the year 2050 (11).

1.2 Heart Valve Replacements

Surgical replacement applying a mechanical or bioprosthetic prosthesis represents the most common therapy for end-stage valvular disease and is a safe and efficient approach. Although current available prostheses demonstrate excellent structural durability (9, 12-14), several limitations remain unsolved including the lack of growth capacity as well as the repair and remodeling properties after implantation into the body. In addition, mechanical valves are well known to have an increased risk of thromboembolic events due to a non-physiological flow pattern and the associated high shear stresses permanently causing erythrocyte damage. Next, a lifelong anticoagulation therapy is required for these patients carrying a risk of spontaneous bleeding and embolism (15). In contrast, bioprotheses, either originating from animals (xenografts) or from human donors (homografts) do not require this lifelong anticoagulation, but are more prone to dysfunctional, degenerative processes requiring high-risk redo operations. Therefore these prostheses are less suitable for middle-aged and younger patients (16) which is also in line with the current clinical guidelines suggesting the use of bioprosthetic valves in patients aged > 65 years and older (12, 13).

While the native heart valve consists of living tissue with the capacity to continuously adapt to the hemodynamic environment (17), none of the above mentioned, artificial prostheses is capable to fully replace the native valvular function due to the lack of these adaptive properties. While in theory, the use of cryopreserved donor heart valves would represent an ideal concept with a low risk for thromboembolic events and infection, the real clinical world appears to be quite different due to the shortness of these prostheses, while the number of indigent patients is permanently increasing (18). Summarising it can be said, these limitations clearly indicate that current therapy options of heart valve replacements are still suboptimal and the ideal concept needs to be developed (11).

The concept of heart valve tissue engineering represents a promising field of research to overcome these limitations and has created substantial hope as well as expectations. The idea of generating a living autologous valve that is not associated with any immune reaction,

thromboembolic events as well as degeneration processes, while offering the potential of growth and remodeling may represent an ideal next generation therapy concept for heart valve replacements.

1.3 The Evolution of Transcatheter Valves

The therapy for valvular heart disease is currently undergoing rapid changes and in addition to conventional valve replacement representing the gold standard since several decades, transcatheter techniques have been introduced into the clinical arena for the treatment of elderly high-risk patients. Current data demonstrate satisfactory hospital outcomes and mid-term results.

While the conventional approach provides very precise and safe suturing of standard valves with low failure rates and excellent proven long-term outcomes (9, 12-14), transcatheter techniques can be performed in off-pump beating-heart fashion that can even be performed without general anesthesia in selected patients. Its long-term safety proven via clinical, randomized trials, it can be assumed that the indications for this minimally invasive procedure will be continuously extended towards younger and less riskful candidates.

Driven by the idea of implanting stented valves into the aortic annulus in the 1990s, the *first-in-man* transcatheter aortic stent-valve implantation was performed by Dr. Cribier in Rouen, France (19) in the beginning of 2002. Two specific routes for implantation have been established since then: the antegrade way via a surgical, direct transapical access, and the retrograde way using a transfemoral catheter-based concept (20, 21). The transapical approach requires a left anterolateral mini-thoracotomy followed by a pledged purse-string suture to enter the apex, whereas the transfemoral method requires an adequate peripheral vascular access and can be performed fully percutaneously (figure 1).

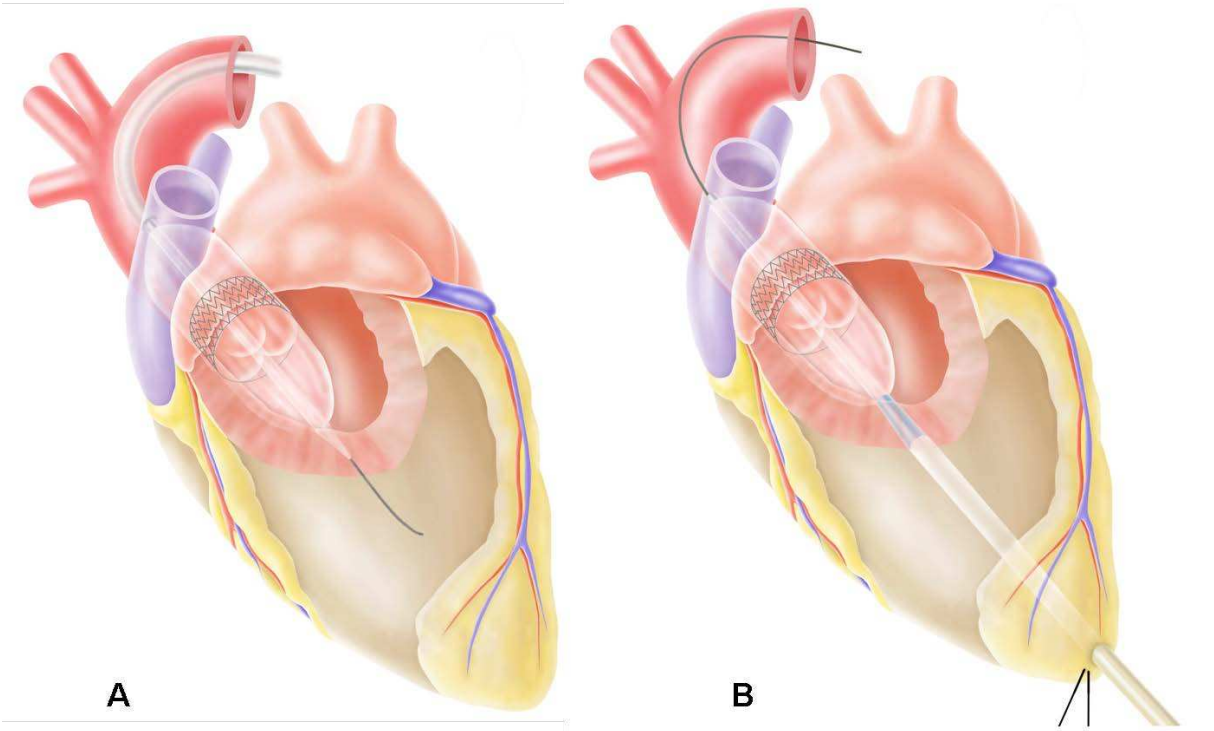

Figure 1: The concept of transcatheter heart valve implantation: *Transcatheter valves can either be implanted via a retrograde, transfemoral approach (A) or via an antegrade transapical approach (B).*

The main advantage of the transapical technique is the independence from concomitant peripheral vascular disease or previous aortic surgery. Furthermore, the delivery system appears to be more “steady” and the procedure itself more “straightforward” and this access potentially reduces the risk of calcium embolism which may occur when a stiff trans-femoral device passes from a peripheral vessel into a diseased aortic arch. The major benefit of the transfemoral approach is the feasibility of a fully percutaneous implantation in conscious patients. However, this only applies to patients with absence of peripheral artery disease and an adequate caliber (>6 mm diameter) of their peripheral vessels not being very tortuous. With regards to the current international guidelines, the presence of peripheral vascular disease, small vessel diameters, tortuous vessels, aortic disease or previous aortic surgery represent the major contraindications for this approach.

Since this breakthrough of the transcatheter technique in 2002, two different suture-less transcatheter aortic stent valves have been developed and were successfully introduced into

the current clinical routine: the Edwards Sapien™ THV stent-valve system by Edwards Lifesciences INC, Irvine, CA, which is available for both the antegrade and the retrograde applications and the CoreValve® system by Medtronic, Minneapolis, MN, which is only available for a retrograde approach. In addition, several other devices are under clinical investigation.

1.4 The Concept of Tissue Engineering

The principle idea of the tissue engineering is the generation of autologous living tissues being equal in architecture and function to their native counterparts. Therefore, a meticulous understanding of the fundamental characteristics of native tissue represents the key factor for the successful generation of native analogous tissue-engineered constructs. In the field of cardiovascular medicine, the in vitro generation of heart valves represents an example of how tissue engineering approaches aim to solve the limitations of current clinical therapy options. Composed of living, dynamic tissue capable of continuous remodeling, native heart valves are permanently adapting to the constantly alternating hemodynamic situation in the circulation (17), while none of the currently available valvular replacement prostheses are capable to fully restore the native function due to the limited long-term adaptive capacity (22). The currently available *state-of-the-art* prostheses in today's clinical routine are well established to show considerable limitations. In particular, these include limited remodeling capabilities and the lack of growth capacity, a major drawback in the setting of congenital applications. In addition, mechanical heart valves are well established to carry an increased risk of thromboembolic events due to high shear stress, nonphysiological flow patterns, and permanent blood damage requiring a lifelong anticoagulation therapy (23), (e.g. warfarin), which is associated with a significant risk of haemorrhagic complications, and thromboembolic incidences. Additional problems may occur in young fertile females when considering the embryo toxicity of warfarin or similar substances. While bioprostheses from xenogenic or homogenic origin do not require anticoagulation, they represent non-viable prostheses prone to degeneration. For this reason, the need for a high-risk re-replacement

normally occurs within 10–15 years and even faster in younger patients due to a higher immunological competence (11, 16).

Tissue engineering technologies may potentially overcome these limitations by creating a living autologous valve replacement that may prevent from an immune reaction, valvular degeneration and thromboembolic events, while offering the significant advantages of growth capacity, remodeling, and regeneration throughout the patient's lifetime. The central dogma of most tissue engineering concepts is the use of temporary, biodegradable supporter matrices in order to support tissue functionality and stability during the engineering process until a sufficient amount of neo-tissue is produced to achieve an adequate physiological function (22). After this in vitro phase, the generated living tissue is implanted into the patient aiming at a further in vivo remodeling process to achieve a sufficient cardiac vascular architecture and function. Significantly influencing the fate of the generated substitute, this in vivo remodeling phase represents a decisive step of a successful engineering concept.

1.5 Strategies in tissue engineering: in vitro or in vivo

Two major tissue engineering strategies have been established to generate living autologous constructs: The in vitro and the in vivo concept. Being the classical approach, the first concept requires a sufficient in-vitro period to generate the ideal native-like construct (22). Briefly, it starts with the isolation and expansion of cells from the patient, followed by the subsequent seeding onto a scaffold material, a defined bio-reactor phase for sufficient in vitro tissue formation and is completed by the final implantation into the patient. This concept, representing the principal approach for heart valve tissue engineering, aims at the complete in vitro generation of the tissue engineered substitute.

In contrast, the second, alternative in vivo approach of heart valve tissue engineering avoids this in vitro tissue culturing period by direct implantation of natural tissue-derived heart valve scaffolds with the idea of potential remodeling and cell in-growth in vivo (24). More recently, and due to the significant advances in the field of stem cell technology, a further in vivo concept has been suggested: Based on the idea of chemo-attractive paracrine pathways, the

seeding of the substitute with autologous stem cells including progenitor and/or mononuclear cells, may lead to the attraction of endogenous cells supporting the remodeling process and the formation of novel tissue (25).

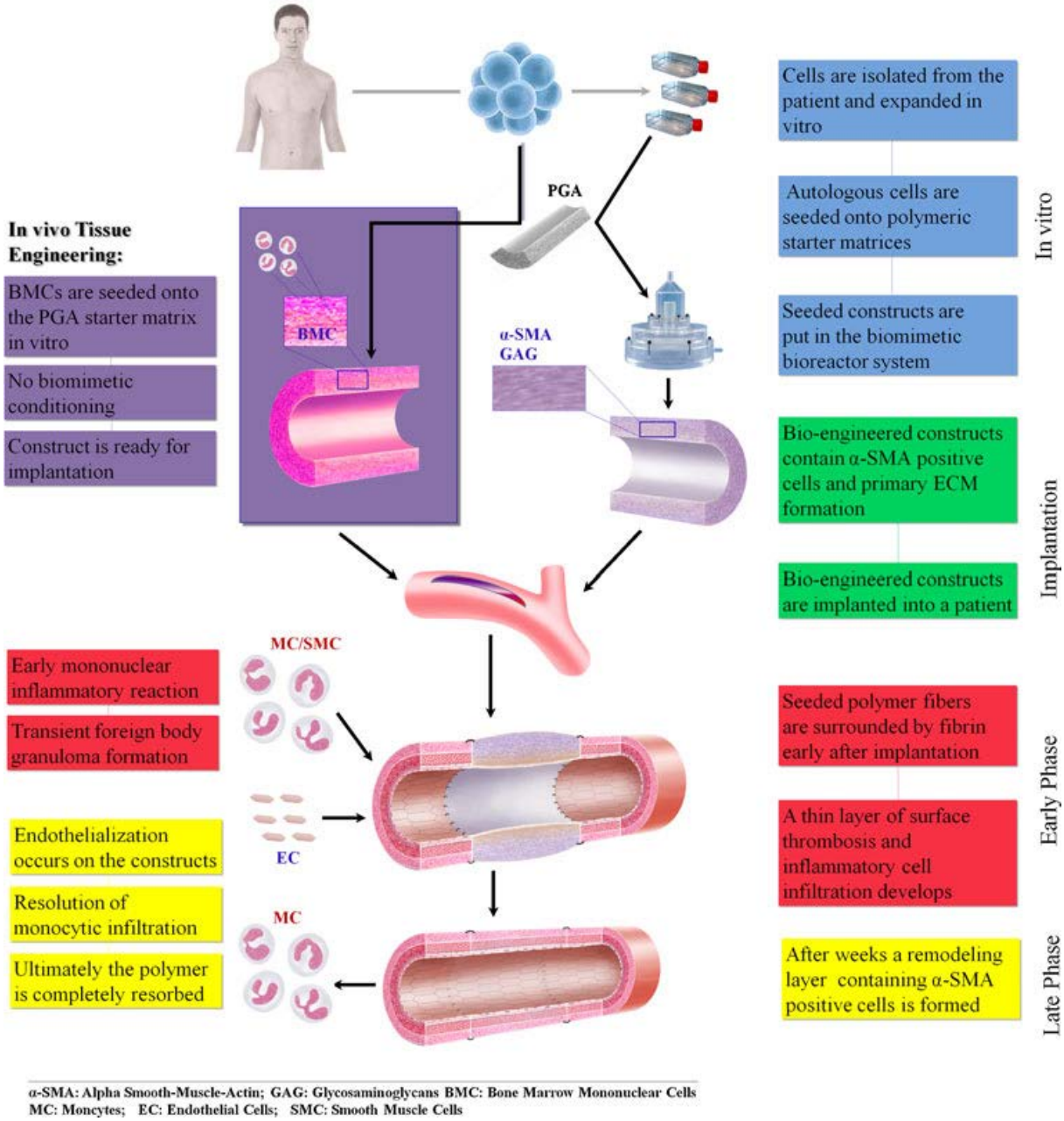

Figure 2: Concept of cardiovascular tissue engineering: *Autologous cells are harvested from the patient and expanded in vitro. When sufficient numbers are reached, cells are seeded onto a biodegradable scaffold. Constructs are either positioned in a bioreactor and conditioned (in vitro approach) or directly implanted into the patient (in vivo approach). After implantation of the tissue-engineered construct, the proposed mechanism of vascular remodeling comprises an early monocyte recruitment to the scaffold with the release of multiple angiogenic cytokines and growth factors. These factors (i.e., VEGF) cause recruitment of host cells, such as MC, SMCs, and ECs, to the scaffold. The invading host cells originate from circulating progenitors and (trans-anastomotic) migration/in-growth of mature vascular cells from adjacent vessel segments. Incoming ECs and SMCs appropriately organize into a mature blood vessel structure on the luminal surface of the scaffold (with the remaining scaffold in the center of the construct). As the scaffold degrades, early monocytes migrate away, leaving behind a remodelled, completely autologous neo-vessel (Weber, Emmert et al; Review; Semin Immunopathol. 2011).*

1.6 Pediatric Implications and the concept of growth

Approximately one out of hundred newborns presents with congenital heart defects often requiring the implantation of prosthetic or homograft constructs including heart valve replacements. The current available prostheses are non-viable substitutes lacking the capacity of growth as well as, repair and remodeling properties. Therefore, a lot of these affected children often require complex and high-risk redo surgery (26).

Based on these disadvantages, alternative strategies remain to be developed. The concept of tissue engineering addressing the fundamental need for growing constructs may represent a promising, next generation approach for future congenital therapy concepts. In the last decade, extensive research has proven the feasibility of autologous tissue engineering concepts for cardiovascular applications such as heart valves (27-29) and blood vessels (30).

Tissue-engineered grafts were successfully applied to the low (31) as well as to the systemic pressure system (32) in animal models and the translation into a clinical setting using human cells has also been demonstrated (27, 33). The group of Shin'oka and colleagues (34) were the first entering the clinical arena and recently reported initial clinical results on vascular autografts generated from human bone marrow cells (35, 36). Following this breakthrough, the field was further pioneered by Hoerstrup and associates who systematically demonstrated the concept of growth in tissue engineered cardiovascular constructs using a long-term, growing animal model. Hoerstrup et al. recently investigated the potential of growth in tissue-engineered living main pulmonary arteries over a period of 100 weeks in a lamb, covering the full growth of this animal model (26).

The animals more than doubled their body weight during the 100 weeks follow up period and concomitantly, there was a significant increase of the mean diameter (30%) and lengths (45%) of the TEVGs in parallel to the native pulmonary arteries indicating sufficient growth

with the animals. Sufficient functionality was demonstrated by regular echocardiography and computed tomography–angiography up to 100 weeks post implantation. In particular, there were no signs of malfunction or degeneration including thrombus formation, calcification, stenosis, suture dehiscence, or aneurysm formation. Next, histology displayed tissue formation reminiscent of the native artery and biochemical analysis revealed cellularity and proteoglycans and increased collagen contents in all of the groups, analogous to those of native vessels (26).

The authors defined this capacity of tissue remodeling and adaptation to the growing cardiovascular system - initiated by in vitro generated tissue engineered vascular grafts – as guided tissue regeneration leading to functional growth. Furthermore, they concluded that this study provides systematic evidence of the capacity of growth in living, functional pulmonary arteries engineered from vascular cells in a full growth animal model highlighting the huge potential of tissue-engineering concepts for congenital applications.

Based on these data and as a next step, selected animals were followed up for up to 5 years. At this point, the initial results could be confirmed with the help of high-resolution computed tomography using a unique 3D CT reconstruction analysis which displayed sufficient and stable wall pressure, shear stress, and flow velocity, in the tissue engineered graft during systole and diastole over the whole follow-up period. In detail, the 3D CT analysis displayed a smooth flow pattern and low wall pressure during systole at all times for up to 240 weeks. The low wall shear stress and the absence of turbulences indicated native analogous vascular performance and absence of degenerative phenomena, such as atherosclerotic calcifications. Moreover, continuous functional growth was confirmed by CT volume measurements, revealing a significant volume increase of the tissue engineered graft starting from an initial volume of 6 ccm 20 weeks after implantation up to 13 ccm after 240 weeks (Emmert & Hoerstrup et al.; unpublished data).

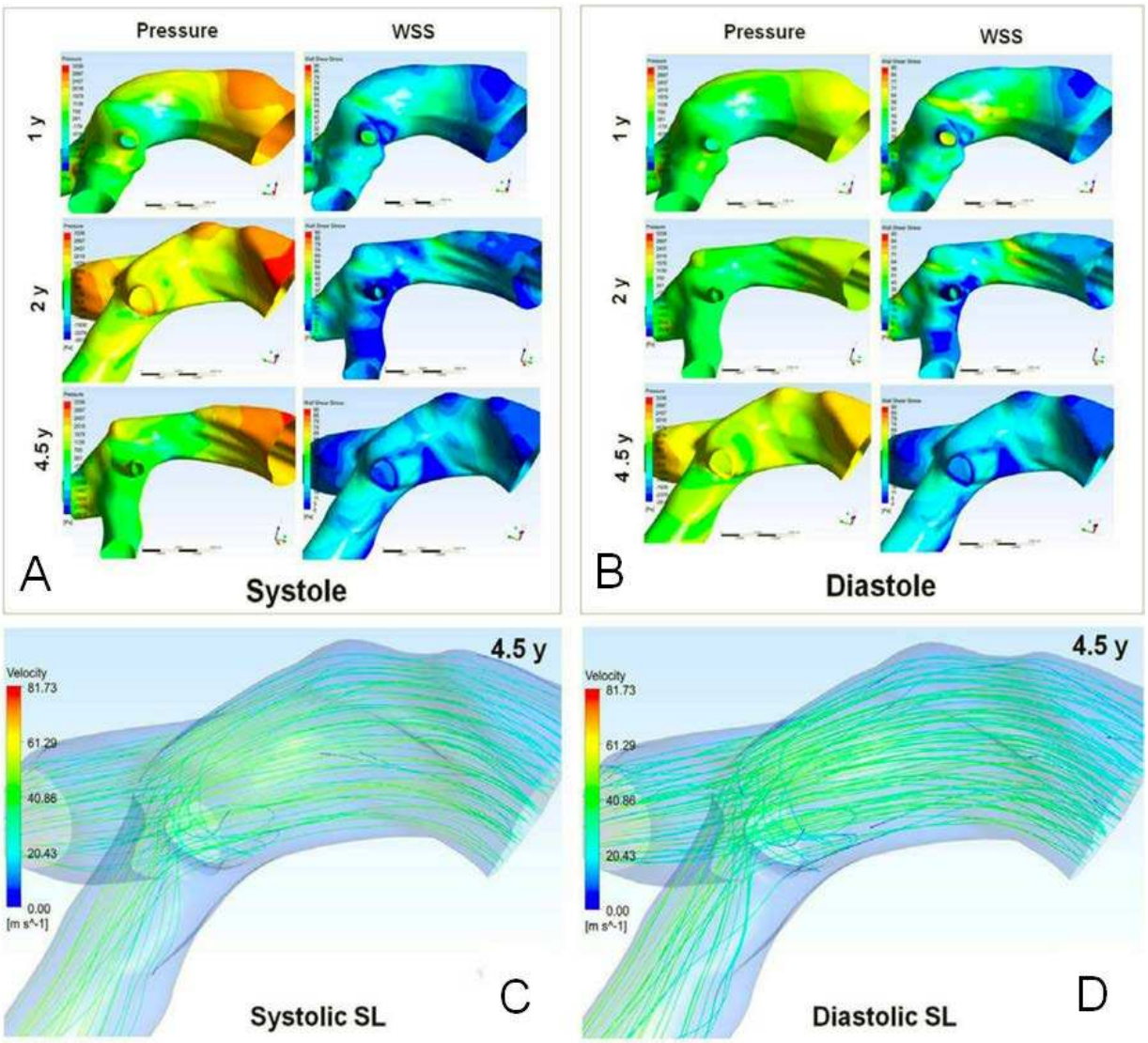

Figure 3: Three dimensional CT reconstruction analysis of wall pressure, shear stress, and flow velocity in the pulmonary-trunc for up to 4.5 years post implantation: *Calculated wall pressure (Pa), wall shear stress (WSS) (Pa) and velocity-coded streamlines (m/s) in the pulmonary trunc during systoly and diastoly of three different sheep, 50, 100 and 240 weeks after implantation of the tissue engineered graft. The flow pattern remains to be smooth with low wall shear stress during the systole in all three sheep, especially after 240 weeks, although the pressure is slightly higher. The low shear stress and the absence of turbulences indicate that no significant wall irregularities, as e.g. atherosclerosis, aneurysms or scars, are present (Emmert & Hoerstrup et al.; unpublished data).*

As it has been reported that follow-up periods in a lamb model can be extrapolated to a four- to fivefold period of time in humans (37), these results represent approx. 20 years in a human demonstrating a first example as to the potential long-term clinical safety of autologous tissue engineering technologies.

1.7 Cell Sources

The selection of the ideal cell type is an important aspect for the long-term success of heart valve tissue engineering (17, 38) and currently different cell sources are under evaluation (22, 29, 38-40).

1.7.1 Vascular cells

The use of vascular cells derived from various saphenous and arterial vessels is an established approach for heart valve tissue engineering (38).In brief, two cell types can be isolated: Endothelial cells (ECs) with antithrombogenic properties and myofibroblasts which are capable to develop extracellular matrix (ECM) (41-43). Following preliminary data in sheep (15, 28, 41-44), human vascular-derived cells revealed sufficient tissue formation after seeding on biodegradable scaffolds (38, 45-47).

1.7.2 Marrow stromal derived stem cells

With regards to clinically relevant tissue engineering concepts, marrow stromal cells (MSCs) may represent an ideal cell source with regards to availability, safety and regenerative potential (15, 27, 48). MSCs were successfully used for the in vitro production of heart valves (27, 48), and also demonstrated sufficient in vivo functionality (49). When compared to vascular cells, these cells can be harvested without the necessity of surgical interventions and can be considered as an easy-to-access cell source. In brief, the usage of MSCs offers several advantages in a) easy harvest by a simple bone marrow puncture (hip puncture or sternal puncture); b) high regenerative potential due to the capacity to differentiate into multiple cell lineages, and c) advantageous immunological characteristics allowing for clinically relevant allogenic scenarios.

1.7.3 Endothelial progenitor cells (EPCs)

Endothelial progenitor cells can easily be isolated from peripheral blood (50) and have been established as source of ECs (50, 51). While representing an easily accessible cell source,

the current focus of research aims at their potential to trans-differentiate into myofibroblast-like cells. Its potential proven, the blood may represent an interesting cell source for heart valve tissue (22).

1.7.4 Umbilical cord derived Cells

The umbilical cord represents a valuable source of different cell types that can be used for heart valve tissue engineering: (1) umbilical cord vein-derived and artery-derived cells, (2) Wharton's Jelly-derived MSCs, and (3) umbilical cord blood-derived EPCs. These cells have been repeatedly demonstrated a high regenerative potential, excellent tissue formation and growth capacities (39, 52-54). The feasibility using umbilical cord derived stem cells to generate different cardiovascular constructs was recently demonstrated (39, 40) suggesting this cell source to be clinically relevant.

1.7.5 Adipose Tissue derived mesenchymal stem cells

Similar to the bone marrow, the adipose tissue also contains mesenchymal stem cells with the potential to differentiate into multiple lineages in vitro (55, 56) and in vivo (57, 58). However, due to the easy access and the high availability, adipose-derived stem cells are likely to represent an alternative stem cell source when compared to MSCs derived from the bone marrow (59).

1.7.6 Prenatal Progenitor Cells

When focussing on pediatric tissue engineering, an ideal concept would comprise an prenatal fetal cell harvest giving the opportunity for tissue engineering processes during pregnancy followed by the implantation instantly after birth (22). Based on this concept, Schmidt et al. successfully generated autologous heart valve leaflets using human prenatal progenitor cells derived from chorionic villi and umbilical cord blood (54). Additionally, the fabrication of living autologous heart valves prior to birth using human amniotic fluid-derived cells could be demonstrated (60, 61).

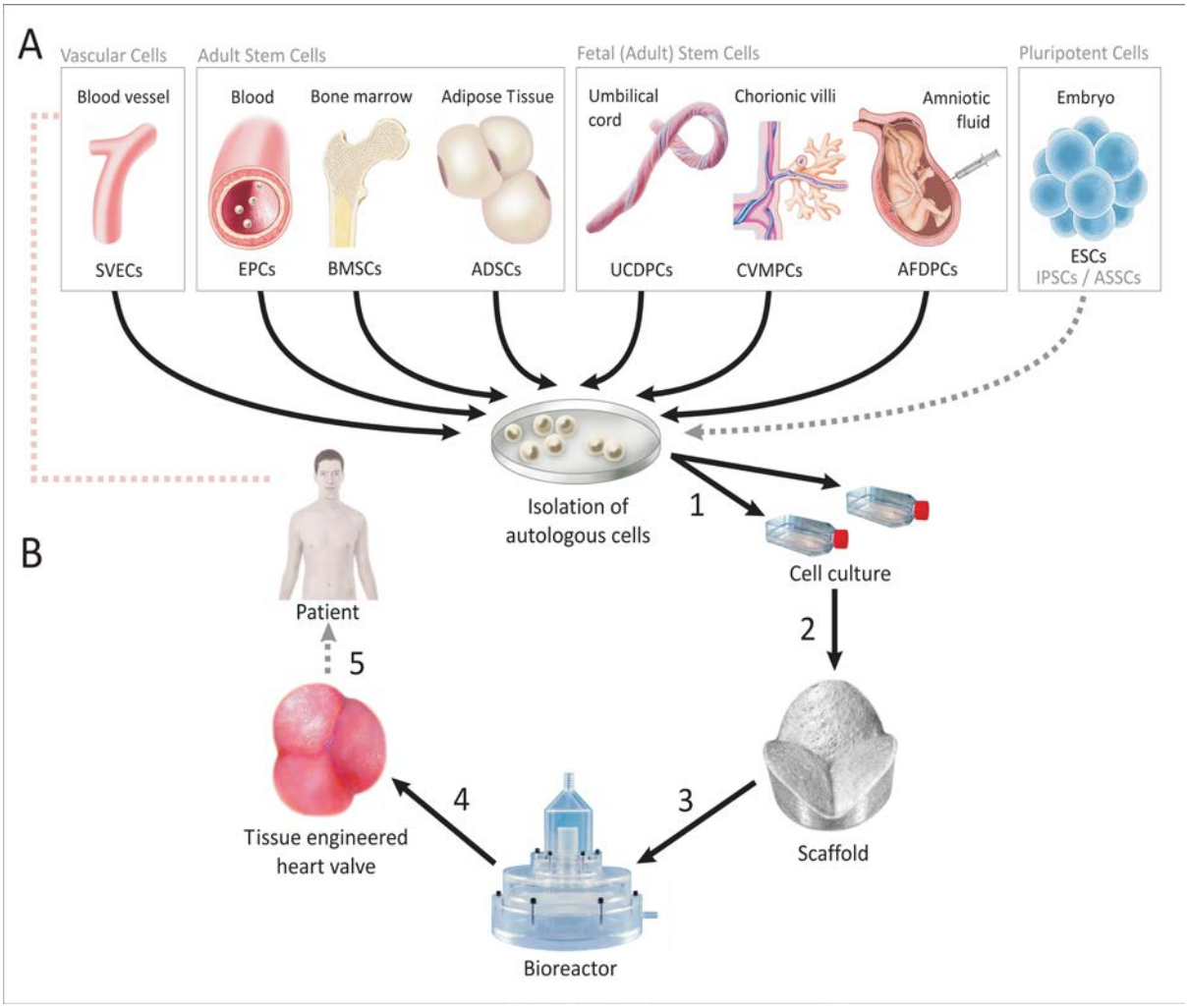

Figure 4: Cell Sources for Cardiovascular Tissue Engineering *Standard vascular endothelial cells (SVECs), Endothelial progenitor cells (EPCs), Bone marrow-derived mesenchymal stem cells (BMSCs), Adipose-derived stem cells (ADSCs), Umbilical cord-derived progenitor cells (UCDPCs), Chorionic villi-derived mesenchymal progenitor cells (CVMPCs), Amniotic fluid-derived progenitor cells (AFDPCs), Embryonic stem cells (ESCs), Adult spermatogonial stem cells (ASSCs) and Induced pluripotent stem cells (IPSCs).* ***(B) Concept of in vitro heart valve tissue engineering.*** *Autologous cells are harvested from the patient and expanded in vitro (1). When sufficient numbers are reached, cells are seeded onto a biodegradable scaffold (2). Constructs are positioned in a bioreactor (3) and conditioned. When tissue formation is sufficient, tissue engineered heart valves (4) are ready for implantation (5) (Weber & Hoerstrup; Bookchapter; Regenerating Heart Valves; Springer 2011).*

1.8 Starter matrices for cardiovascular tissue engineering

The development of biocompatible starter matrices and scaffolds for heart valve tissue engineering comprises of two main routes: (a) biological matrix materials and (b) a fully synthetic scaffold (22, 62).

In principal, the design of a scaffold should be adapted to the native counterpart as exactly as possible. Next, it should support cellular growth including cell-to-cell interaction and neo-tissue formation displaying an organ specific extracellular matrix. Furthermore, it should be

capable to cope with the mechanically complex cardiovascular environment. These mentioned key aspects are crucial for the success of the tissue-engineered constitutes, regardless of the material of the scaffold or the matrix.

The surfaces of these starter matrices must provide sufficient biocompatibility including a significant potential of cellular in-growth, as well as an optimized degradation rate for cellular expansion (63). Based on these specific requirements, numerous concepts to identify the ideal scaffold material have been developed and are currently under investigation including the creation of biological (64) and synthetic scaffold materials (65) which can further be subdivided into subgroups (22): these include native tissue-derived ECM scaffolds (66), polymeric scaffolds (67-70), biological-polymeric hybrid scaffolds (71-73), and collagen or fibrin gel scaffolds (74-77).

Although significant advances have been made in all of these concepts, the application of polymeric scaffolds has turned out to be the most interesting approach in the field of heart valve tissue engineering approaches.

In general, the application of polymeric scaffolds for different tissue engineering strategies has already been extensively elucidated (65). With regard to state-of-the-art tissue engineering an ideal scaffold matrix has to be at least 90% porous (78) which is essential for cell growth and nutrient supply. Additionally, the scaffold material should also meet the mechanical criteria of a native heart valve and should display a cell-favourable surface chemistry (65). Next, the degradation rate of the matrix should be controllable and adaptable to the rate of neo-tissue formation (17, 79). Table 1 summarizes a compilation of different synthetic polymers which are under evaluation as potential starter matrices in the field of heart valve tissue engineering.

As one concept, the family of aliphatic polyesters including polyglycolic acid (PGA), polyglactin (PG), and polylactic acid (PLA) has been used to create single heart valve

leaflets. However, the major limitations of these aliphatic polyesters - when used as sole material - are their initial stiffness, thickness, and non-pliability making it complicated to generate complete tri-leaflet heart valves (22).

In another concept, polyhydroxyalcanoate (PHA) and poly-4-hydroxybutyrate have been used to generate tri-leaflet heart valve conduits (47, 80) and while these materials Are easily transferable to any desired shape (81) and provide excellent thermoplastic properties, their low degradation rate can be considered as a major limitation.

Furthermore, the combination of aliphatic polyesters and PHAs has been applied as a potential alternative (28, 82) and particularly the approach with P4HB coated PGA may be efficient. Combining the thermoplastic properties of P4HB and the excellent porosity of PGA seems to be a promising approach for the fabrication of tissue engineered heart valves (27, 28, 61, 82-84).

Scaffold	Construct
Lactide acid and P-caprolactone and PGA/PLLA	Vascular autograft
PEUU and PEEUU	Vascular patches
PGA	Vascular patches/graft
P4HB	Vascular graft
PHA	Vascular graft
PHO	TEHV
PGA/P4HB	THEV

Table 1: Examples of polymeric starter matrices used for cardiovascular tissue engineering *(Weber, Emmert et al; Review; Semin Immunopathol. 2011)****;*** ***PGA*** *polyglycolic acid;* ***PHA*** *polyhydroxyalkanoate;* ***PHO*** *polyhydroxyoctanoate;* ***PEUU*** *poly(ester–urethane)urea;* ***PEEUU*** *poly(ether–ester–urethane)urea;* ***PLLA*** *polylactic acid;* ***P4HB*** *poly-4-hydroxybutyrate;* ***TEHV*** *tissue-engineered heart valve*

1.9 The implant-mediated inflammatory response: the key to tissue remodeling?

The idea of in vivo healing of tissue-engineered constructs can be considered as a continuous, multi-factorial process. After the initial blood-material interactions this cascade consists of an initial acute inflammation which is then followed by several repair processes (figure 5): In brief, immediately after implantation, phagocytic cells originating from the microcirculation (such as neutrophiles and monocytes which appear to play a key role in the

healing and remodeling process) enter the interface between the implant surface and the injured native tissue. The period of acute inflammation (up to several weeks in humans) includes the initial phagocytic removal of debris due to trauma, which is then followed by a phase of appropriate signalling to shift from inflammation to repair, regeneration and remodeling of the tissue (22). Mendelson and colleagues have recently suggested that the success of in vivo remodeling of tissue-engineered substitutes is decisively influenced by two main processes: (A) The formation of an atypical vascular response to injury at the luminal surface, including intimal thickening, pannus formation, and neointima development, and (B) deep tissue biomaterial-associated effects of foreign body reaction, granulation, tissue formation, and fibrosis forming a media-like structure (85).

On the cellular level, monocytes appear to play a crucial role in the different steps of the healing cascade. After the initial phase of acute inflammation, they are detectable in the implanted construct. It has also been reported that the monocyte chemotactic protein (MCP-1), a potent monocyte-attracting cytokine, is released by activated neutrophils at the site of implantation indicating to represent a key factor within the early remodeling period, which is mainly characterized by chemotactic immune cell infiltration (86).

Next, monocytes seem to play an important role in the entire remodeling process as they remain in the implanted scaffold until it has fully degraded (22). A recent study of Hoerstrup et al. demonstrated a heterogeneous mononuclear inflammation in the areas of residual polymer fibers for up to two years after implantation of a tissue-engineered graft (26). Moreover, monocytes produce a wide range of cytokines (i.e., MCP-1/CCL-2, IL-6, IP-10), growth factors, and proteases which are required for vascular cell proliferation/migration and appropriate vascular remodeling (85, 87, 88). Furthermore, they express extracellular matrix remodeling proteases (i.e., MMPs), cytokines characteristic of the innate immune response (IL-1α, IL-1β, IL-6, IL-10, and TNFα) and cell adhesion (ICAM-1, VCAM-1) (22).

Apart from the decisive role of MCP-1 for monocytic attraction and despite a successful proof-of-concept study attaching MCP-1-releasing biodegradable microparticles to the scaffold matrix in order to mimic the chemo-attractive properties of seeded cells in vivo, the exact pathways explaining these remodeling mechanisms remain unclear. However, for the development of strategies to modulate early inflammatory responses and to increase tissue remodeling, the complete understanding of these underlying cascade is crucial and has to be elucidated (22, 89).

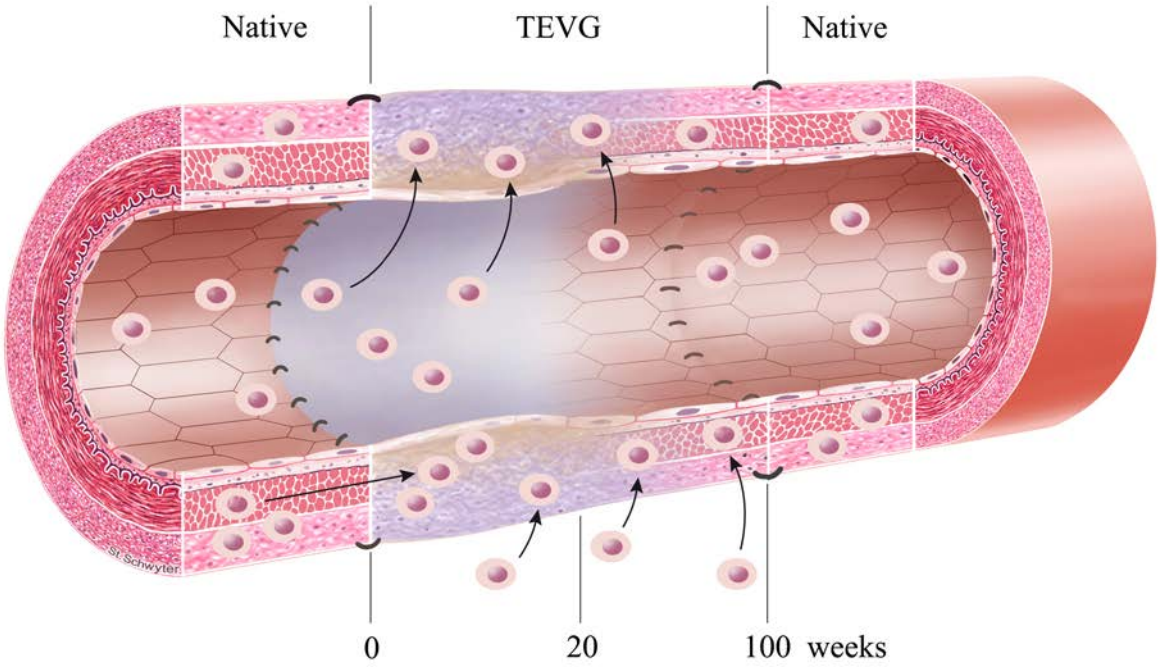

Figure 5: The concept of remodeling and functional healing response: *Schematic diagram depicting the concept of remodeling and functional healing response in a tissue engineered graft model: After implantation of a tissue engineered vascular graft (TEVG), a functional healing response occurs (0-20weeks), which is accompanied by a transient inflammatory response, cellular in-growth and repopulation of endogenous cells into the TEVG. A pannus containing a-SMA and desmin cells has formed at the luminal surface (neo-intima) and granulation tissue evolving into fibrous tissue containing glycosaminoglycans and collagen. These cells originate from different sources and approach the TEVG via different mechanisms including the trans-anastomotic in-growth (i), blood derived cell immigration (ii), or adventitia derived cell migration (iii). Whereas the interface between the TEVG and the native vessel is still detectable within the period of 20-100 weeks it is equalized after a 100 weeks and fully integrated and indistinguishable after 240 weeks in vivo indicating completion of tissue remodeling beyond 100 weeks (Emmert & Hoerstrup et al; unpublished concept figure).*

1.10 Aim of Habilitation Thesis

This habilitation thesis summarizes the systematic development of translational, clinically relevant tissue engineered heart valve concepts by combining the use of sophisticated stem cell approaches, *state-of-the-art* tissue engineering protocols and minimally invasive valve implantation techniques.

2. Summary of Publications

(full publications are available in the appendix, page 62)

2.1 Minimally-Invasive Implantation of Living Tissue Engineered Heart Valves - A Comprehensive Approach from Autologous Vascular Cells to Stem Cells

Schmidt D, Dijkman PE, Driessen-Mol A, Stenger R, Mariani C, Puolakka A, Rissanen M, Deichmann T, Odermatt B, Weber B, ***Emmert MY****, Zund G, Baaijens FP, Hoerstrup SP Journal Am Coll Cardiol. 2010 Aug 3;56(6):510-20.*

In this article, for the first time, the principal feasibility of merging tissue engineering and minimally-invasive valve implantation techniques using vascular cells and adult stem cells was successfully demonstrated.

While the feasibility and functionality of tissue engineered heart valves (TEHVs) implanted by conventional surgical procedures have been demonstrated by in vivo experiments and initial clinical trials (27-29, 55, 65, 84), a clinically relevant heart valve tissue engineering concept would ideally comprise both, minimally-invasive techniques for cell harvest and valve implantation. Therefore, the aim of this study was to demonstrate the feasibility of combining the novel heart valve replacement technologies of: 1) tissue engineering; and 2) minimally-invasive implantation techniques based on autologous cells and composite self-expandable biodegradable biomaterials.

Tri-leaflet heart valves fabricated from biodegradable synthetic scaffolds, integrated in self-expanding stents and seeded with autologous vascular or stem cells (bone marrow and peripheral blood), were generated in vitro using dynamic bioreactors. Subsequently, the tissue engineered heart valves (TEHV) were minimally-invasively implanted as pulmonary valve replacements in juvenile sheep. In addition to the in vivo functionality assessed by

echocardiography and angiography up to 8 weeks, the tissue composition of explanted TEHV and corresponding control valves was evaluated.

The implantation procedure was uneventful in all animals with regards to perioperative morbidity or mortality. Confirmed by fluoroscopy, all valves were deployed successfully at the targeted site and no migration of the TEHV or paravalvular leakage was observed. Transthoracic echocardiographic demonstrated sufficient valvular function and revealed an initial mean pressure gradient of 30 mm Hg which was constantly decreasing to 10 mm Hg. Two study animals displayed minimal regurgitation which remained unchanged over the whole period of implantation time. Histology revealed layered neo-tissues with endothelialized surfaces. Quantitative extracellular matrix analysis at 8 weeks showed higher values for deoxyribonucleic acid, collagen, and glycosaminoglycans compared to native valves. Mechanical profiles demonstrated sufficient tissue strength, but less pliability independent of the cell source after 4 weeks in vivo.

For the first time and in a translational, clinically relevant setting, the principal feasibility of combining stem-cell based, autologous tissue engineering concepts and minimally-invasive valve replacement techniques was demonstrated.

2.2 Tissue engineered vascular graft remodeling in a growing lamb model: expression of matrix metalloproteinases

Cummings I, George S, Kelm J, Schmidt D, ***Emmert MY****, Weber B, Falk V, Zünd G, Hoerstrup SP*

Eur J Cardiothorac Surg. 2011 Apr 27. [Epub ahead of print]

As future clinical applications of tissue engineering require a meticulous understanding of the underlying mechanisms of neo-tissue formation and matrix remodeling, in this article the specific role of matrix metalloproteinases (MMP) with regards to matrix remodeling, tissue homeostasis and functional growth was investigated.

Matrix metalloproteinases (MMPs) are zinc-dependent peptidases broadly classified according to their substrate-degradation capabilities and were found to contribute to extracellular matrix remodeling more than fifty years ago. Additionally, MMP-related processes are expected to play a key role in tissue homeostasis and functional growth. Synthesized in the latent form as Zymogens, they are secreted as pro-enzymes (pro-MMPs), before they are activated via a process known as the cysteine-Zn+ switch which enables proteolytic activity (90, 91). In summary, smooth muscle cells produce pro-MMP-2 in the wall of normal arterial walls and in culture, while active MMP-2 and pro- and active MMP-9 are absent. In contrast, diseased vessels undergoing remodeling processes generate active MMP-2 and MMP-9 (92-95). Therefore the focus was on the expression of the gelatinases MMP-2 and-9. MMP-2 has been demonstrated to degrade native type IV, V, VI, and X collagen as well as denatured collagen, proteoglycans, elastin, and growth factors. MMP-9 degrades gelatin, an irreversibly hydrolyzed collagen, and is expressed following injury or inflammatory stimulation (96).

Using a previously published growing lamb animal model elucidating the concept of growth (26), tissue-engineered autologous living pulmonary arteries were analyzed as to MMP

profiles including MMP-2 and -9 expression as well as microstructure and tissue composition, and were compared with native arterial tissue. Briefly, these tissue engineered vascular grafts generated from biodegradable scaffolds seeded with autologous vascular cells were cultured in static and dynamic in vitro conditions. Thereafter, they were implanted as pulmonary artery replacements in lambs and followed up for 2 years.

Gelatin gel zymography to detect MMP-2 and -9 was done and collagen content quantification was performed. Latent (pro) and active MMP-2 and -9 were detected. Comparable levels of active MMP-9 and pro-MMP-2 were identified in both, static and dynamic cultures, while higher levels of active MMP-2 were detected in dynamic cultures. Whereas the expression of MMP-2 and -9 was very low in native grafts, it but was increased in the implanted tissue engineered vascular grafts. Pro-MMP-9 was expressed 20 weeks after implantation persisting up to 80 weeks post implantation. The in vitro analysis revealed increased levels of collagen after dynamically conditioned tissue engineered grafts when compared to their statically conditioned counterparts. Next, the collagen content of the engineered gtrafts was also higher when compared to the native vessel. Based on these results, it was concluded that MMPs are up-regulated in vitro by dynamic culture conditions and may contribute to enhanced matrix remodeling, native analogous tissue formation and functional growth of tissue engineered grafts in vivo.

Furthermore, the presence of elevated MMP activity even in the long-term grafts suggests a sustained remodeling activity maintaining functionality and homeostasis of the tissue. Based on a more meticulous understanding of matrix remodeling, state-of-the-art imaging modalities may support non-invasive tracking of MMP activity in clinical settings to define clinically relevant tissue quality criteria that may contribute to the safe translation of tissue-engineering technologies to the clinical arena (97). Next, a more detailed insight into the remodeling pathways involving MMPs may support the specific production of design intelligent scaffold materials supporting remodeling phenomena within tissue-engineered constructs by attaching transcription factors and/or cytokines to the starter matrices.

2.3 Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates

Emmert MY*, *Weber B*, Scherman J*, Gruenenfelder J, Verbeek R, Bracher M, Black M, Kortsmit J, Franz T, Schoenauer R, Baumgartner L, Brokopp C, Agarkova I, Wolint P, Zund G, Falk Zilla P, Hoerstrup SP.*

*Eur Heart J. 2011 Mar 17. [Epub ahead of print]; *contributed equally*

Following the demonstrated feasibility of merging in-vitro tissue engineering concepts with minimally invasive implantation techniques (49) and based on the principle idea of an in vivo remodeling approach, the aim of this study concept was an even more clinically relevant setting. Using a non-human primate model being well comparable to humans, the fabrication of marrow stromal cell-based, autologous, living tissue engineered heart valves (TEHVs) and direct minimally invasive implantation in the same intervention (one step procedure) as pulmonary valve replacements was tested (figure 6).

Trileaflet heart valve scaffolds were generated andintegrated into self-expandable nitinol stents. Bone marrow was aspirated from the sternum of adult Chacma Baboons and mononuclear cells were obtained by centrifuging the samples on a histopaque density gradient seeding of the BMCs onto the stented heart valve scaffolds (8+3 ×106 cells/cm2). This was performed using fibrin as a cell carrier. The isolation of BMCs revealed 175+82 × 106 cells per animal for seeding, while cellular viability was in excess of 95%. Isolated primate mononuclear cells stained positively for common leucocytic antigens including CD45, CD44, and CD15 and flow cytometric analysis of the marrow mononuclear cells comprised distinct leucocytic cell populations. Primate bone marrow aspirate contained CD34+ haematopoietic stem cells as well as mesenchymal stem cells staining positive for CD44, CD90, CD146, and CD166, while being negative for CD45 and CD34, representing a common staining pattern for MSCs. The differentiation potential of isolated primate MSCs was confirmed exemplarily using pre-adipocytic and osteoblastic differentiation assays.

The transapical implantation procedure was successful in all animals and in five out of six cases the valves were optimally deployed in the orthotopic valvular position, whereas one valve was positioned supravalvularly thereby not excluding the native valvular leaflets. One study animal with an orthotopically implanted valve suffered perioperatively from coronary perfusion complications which were not related to the TEHV functionality and was terminated 12 hours post implantation. The positioning of the TEHV was confirmed intra-operatively using TEE and fluoroscopy. Importantly, neither migration of the TEHV nor paravalvular leakage was observed, while sufficient valve functionality was demonstrated. The mean crimping time of the TEHV, from insertion into the application system until surgical deployment, was 17+8 min and the mean duration of the entire procedure, from bone marrow harvest until valve delivery, was 118+17 min.

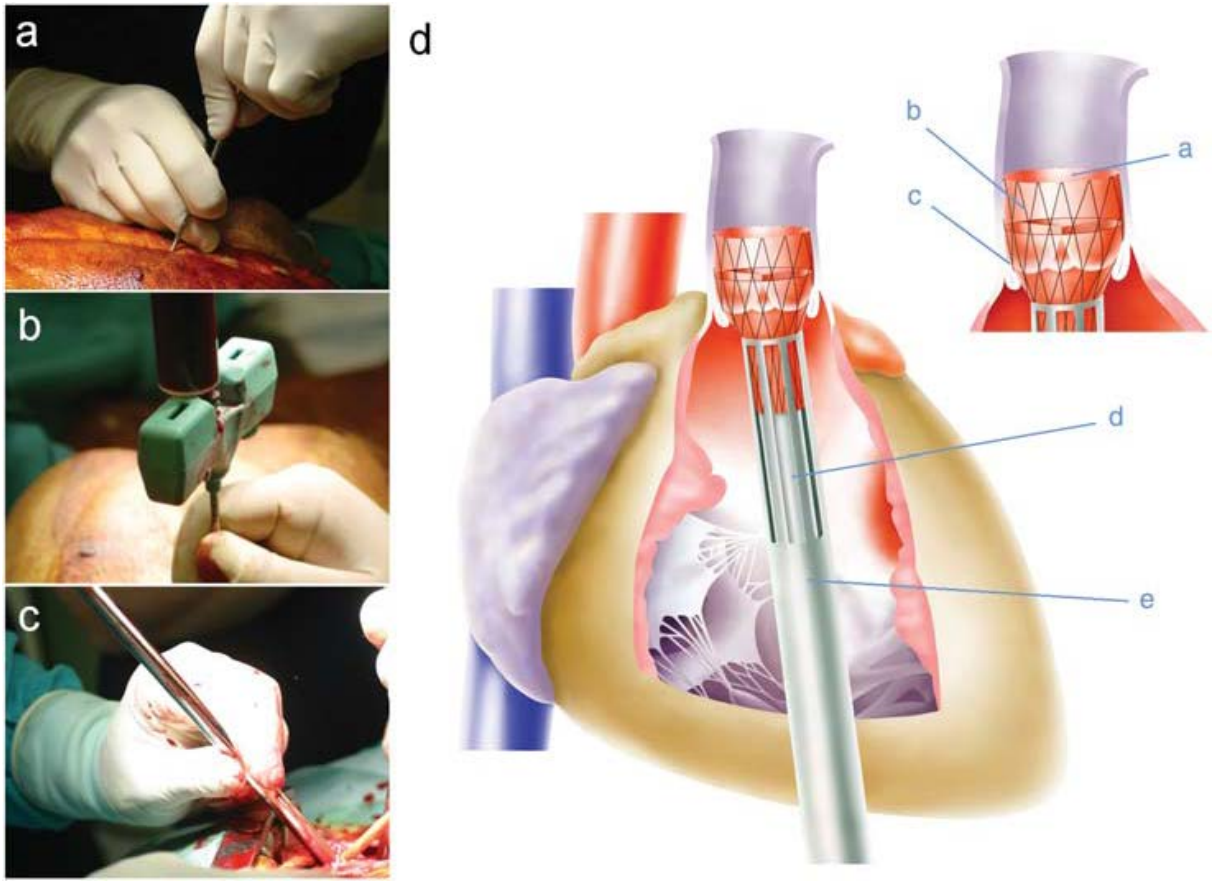

Figure 6: Concept of One-Step Procedure for Minimally invasive tissue engineered heart valve implantation: *After sternal bone marrow puncture (A) and aspiration of fluid (B), the tissue engineered heart valve was loaded into the delivery device (C), inserted into the right ventricle (D) and deployed in the pulmonary position under sonographic and fluoroscopic guidance. The crimped tissue engineered heart valve (a), integrated into the nitinol stent (b), was carefully deployed into the pulmonary position (c) by slowly advancing the inner pusher (d) into the sheath system (e).*

While sufficient valve functionality and absence of any paravalvular leakage could be demonstrated throughout the follow-up period (4 weeks), weekly TTE / TEE controls displayed a slight but not significant increase in transvalvular peak pressure starting around 25+ 7 mmHg (TEE) perioperatively, up to 30+7 mmHg after 3 weeks (TTE). The harvest of the early TEHV (12 h) displayed an intact leaflet structure dominated by a fibrin-coated PGA scaffold matrix without any sign of thrombus formation. The explantation of the remaining TEHV 4 weeks post implantation showed constructs that were well integrated into the adjacent tissue-by-tissue covering the stent margin and encircling stent strut endings. The valvular leaflets of the four orthotopically positioned constructs presented as pliable, well-defined cusps and although the leaflets seemed to be shortened in radial diameter, sufficient co-aptation could be confirmed throughout the entire experimental period (figure 7). Interestingly, in the haemodynamically non-fully loaded TEHV deployed in the supravalvular position the leaflet structure was almost entirely missing.

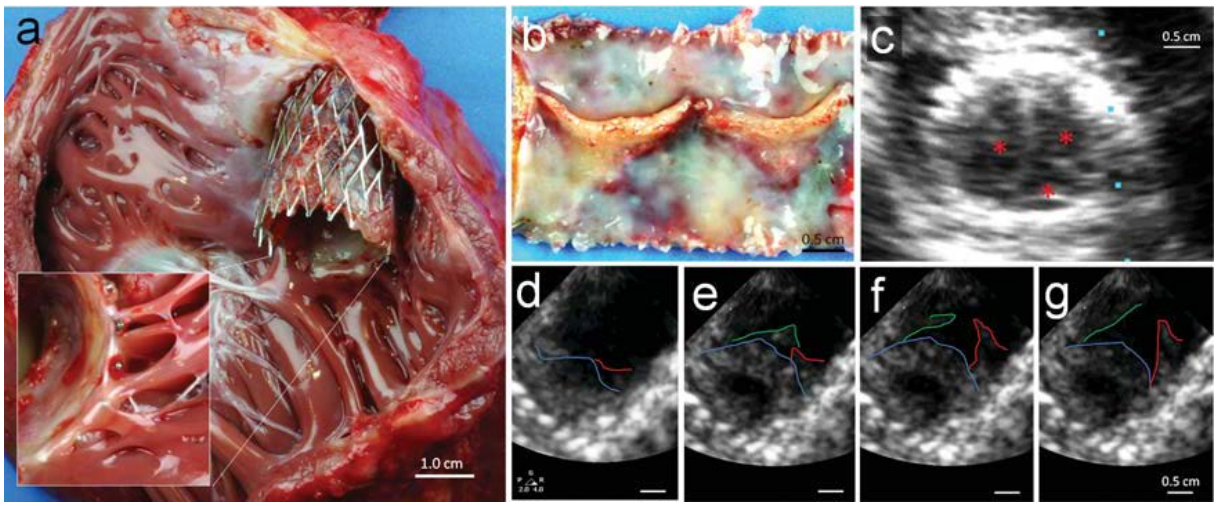

Figure 7: Explant analysis of tissue engineered heart valves. *After 4 weeks in vivo the stented constructs were well integrated into the adjacent tissue (A). Orthotopical tissue engineered heart valves (B) presented with a cusp-like leaflet structure, with shorter leaflets than native controls. In a final transoesophageal echocardiography-assessment the leaflet co-aptation (C; asterisk indicates leaflets) as well as opening movements of all three leaflets could be visualized (D–G).*

Scanning electron microscopy displayed that the surface of the early explants (12 h) was mainly characterized by previously seeded mononuclear cells embedded into a fibrin matrix densely covering the entire scaffold. Analysis of the 4-week explants revealed that three of the orthotopically implanted leaflets showed already a mature structure, characterized by a

well-defined endothelial lining on the conduit wall as well as partially on the valvular leaflets. In several spots these endothelial coverages were confluent and not distinguishable from native primate or human valvular endothelium.

Next, the Haematoxylin–eosin staining of the explants demonstrated layered tissue morphology, characterized by a central core of non-degraded PGA matrix surrounded by dense tissue formation on the luminal as well as on the vascular side. Masson's Trichrome and eVG staining confirmed the presence of collagen predominantly in the outer layers of the conduit wall and the leaflet (figure 8).

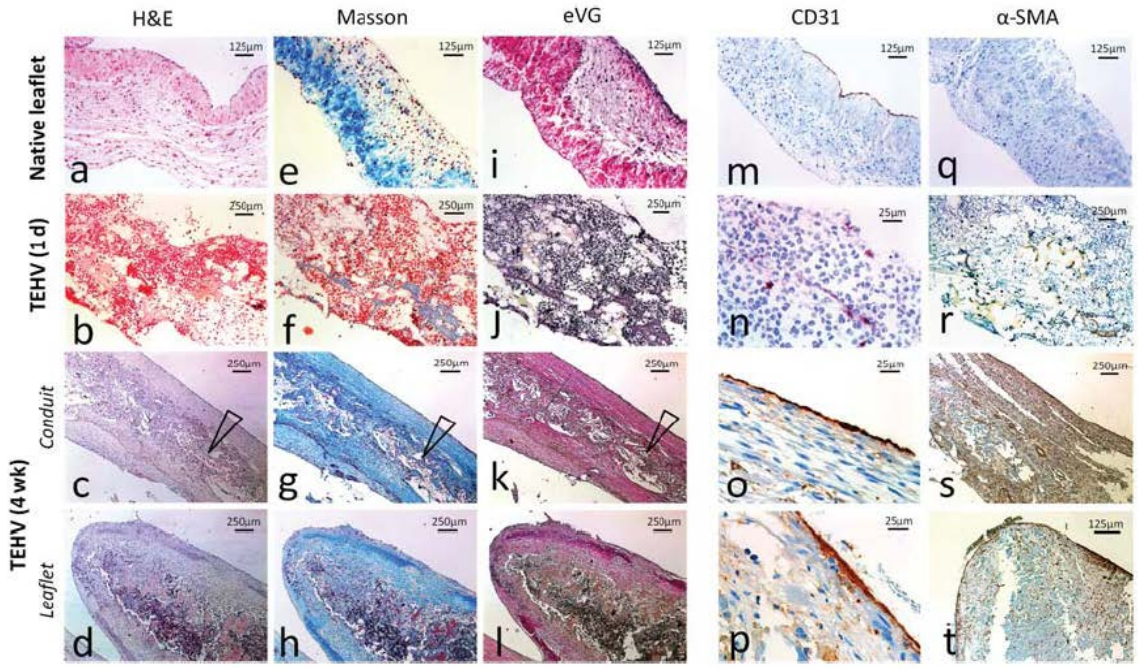

Figure 8: Histology of the explanted living tissue engineered heart valves. *Explanted tissues showed layered tissue architecture visualized by haematoxylin–eosin staining (A–D). In Masson's Trichrome staining (E–H) and Elastin-van-Gieson staining (I–L) collagen fibres were predominantly found in the outer tissue layer of the conduit and leaflets. Cells of the conduit (O) and leaflet (P) surface layer expressed CD31 (M–P). a-SMA expression (Q–T) was detected in the outer layers of the conduit (S) and the leaflet (T), but was missing in the proximal leaflet region. Arrows indicate reminiscent scaffold material.*

As confirmed by SEM, the surface of the conduit was covered by a confluent endothelial layer in large areas of the constructs (figure 9). Cells of the constructs' surface layer expressed CD31 and vWF, resembling the staining pattern of native valve endothelial cells. The early (12 h) as well as the late (4 weeks) explants stained positively for CD68, indicating a distinct monocytic infiltration and continuous remodeling. Cell tracking analysis using

CFSE-dye and confocal microscopy revealed no signal within the explanted tissue, indicating that most of the seeded BMCs were not present after 4 weeks in vivo.

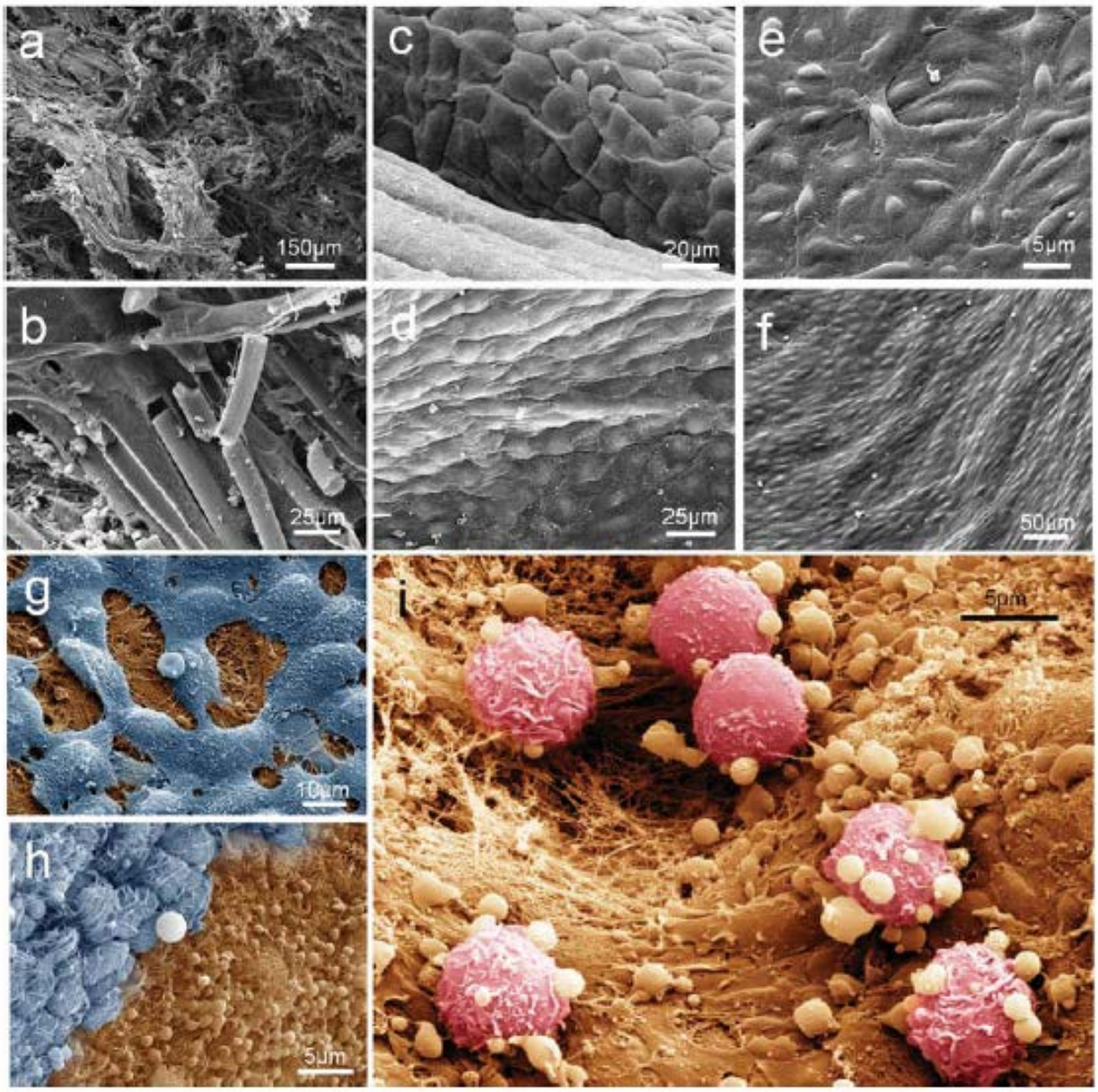

Figure 9: Scanning electron microscopy *of the polyglycolic acid–poly-4-hydroxybutyrate scaffold (A and B), primate (C), and human (D) control leaflets. In most areas the surface of the 4 week explants showed confluent (E and F) or initial (G) endothelial coverage. In some areas the surface remodeling was still evident involving thrombocyte attachment (H) and leucocyte attraction (I).*

Quantitative extracellular matrix (ECM) analysis of the TEHV explants showed that the GAG as well as the DNA content of the constructs' leaflets was higher compared with native tissue (GAG 140.28+36.17%; DNA 168.10+87.07%), indicating high cellular infiltration as well as ECM remodeling.

Based on these results, the study showed for the first time the feasibility of merging heart valve tissue engineering using a low-invasive stem cell source, with a minimally invasive implantation technology combined in a single intervention. Autologous bone marrow derived mononuclear cells can be isolated in sufficient numbers either from the sternum or the hip thereby enabling immediate re-implantation as autologous TEHV. Its long-term functionality proven, the presented one-step approach suggests a highly clinically relevant concept and may represent a significant step towards the routine utilization of TEHVs. These first results are promising and demonstrate the feasibility of using BMCs for the fabrication of TEHV. Moreover, utilizing the body's natural abilities to regenerate TEHV in vivo, may greatly simplify, and improve the clinical feasibility of the autologous cell-based TEHV approach.

2.4 Transapical Aortic Implantation of Autologous Marrow Stromal Cell-based Tissue Engineered Heart Valves – First Experiences in the Systemic Circulation

Emmert MY, *Weber B, Behr L, Frauenfelder T, Brokopp CE, Grunenfelder J, Falk V, Hoerstrup SP*

JACC Cardiovasc Interv. 2011 Jul;4(7):822-3.

After the feasibility of catheter based implantation of a marrow stromal cell based, autologous tissue engineered heart valve in a one-step intervention was proven (98), as a next step this concept was tested in the high-pressure systemic circulation.

While the concept of minimally invasive tissue engineered heart valve implantation has only been proven for the low-pressure system of the right heart so far, the idea of this proof-of concept acute study was to challenge the high-pressure circulation. The feasibility of transcatheter tissue engineered orthotopic aortic valve replacements in a one step intervention would be the ultimate goal to broaden the future potential of tissue engineered heart valve (TEHV) approaches and would define the next generation concept with a vast clinical impact addressing a large cohort of patients. However, when taking the more complex anatomic conditions due to the coronary perfusion into account, as a first step, potential non-TEHV related, technical complications were avoided by implanting the valve in a supra-coronary position.

Once more, within a one-step intervention tissue-engineered, living heart valves generated from biodegradable scaffolds and seeded with autologous bone-marrow derived mononuclear cells, were integrated into self-expanding nitinol stents (20mmx30mm) and transapically delivered into the descending aorta (distal to the brachiocephalic trunk) and brachiocephalic trunk of sheep. Prior to implantation, native valve insufficiency was created by applying the *Hufnagel* procedure an accepted technique to induce heart valve incompetence (99) (figure 10). After successful deployment, the optimal positioning and

valve functionality were confirmed using fluoroscopy, computed-tomography (CT) and trans-esophageal echocardiography. The crimping-time of the TEHVs was 15±2 minutes and the overall duration of this one-step intervention from the preparation of cells to the successful delivery was approximately two hours. In detail, the TEHVs demonstrated excellent in- vivo functionality with well defined leaflets showing sufficient coaptation. These results were confirmed by post-mortem analysis displaying fully intact TEHVs without any signs of leaflet-rupture, microstructure damage or thrombus formation.

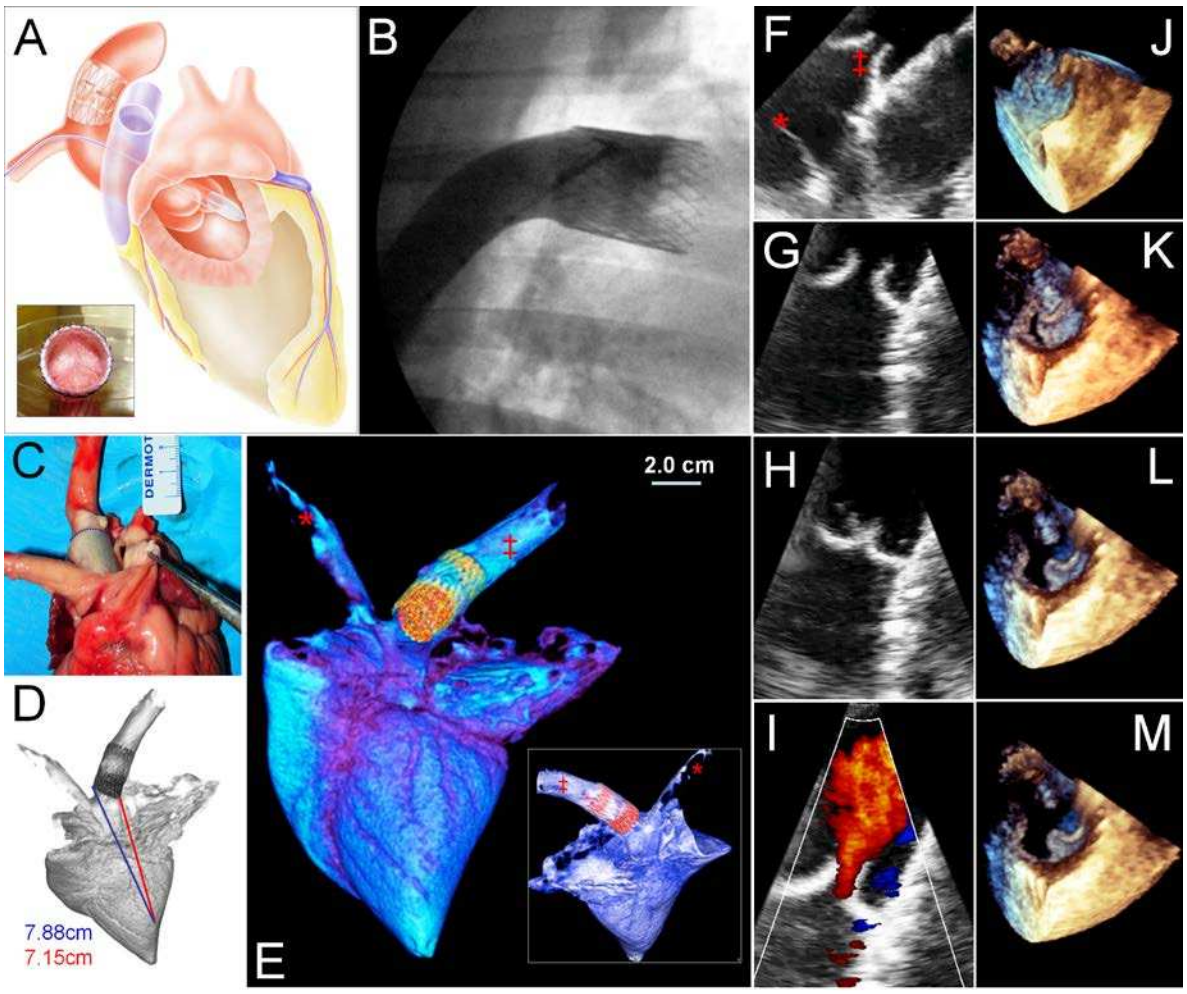

Figure 10: Concept of One-Step Procedure for minimally invasive tissue engineered heart valve implantation into the systemic circulation: *Within one procedure tissue-engineered, living heart valves (TEHV) fabricated from biodegradable scaffolds seeded with autologous bone-marrow derived mononuclear cells (a/inset), were integrated into self-expanding nitinol stents (20mm x 30mm) and transapically delivered into the descending aorta (distal to the brachiocephalic trunk) of sheep (a). Native valve incompetence was created by applying the Hufnagel procedure prior to implantation (a). After successful deployment (b-c), valve-function and optimal positioning were confirmed using fluoroscopy (b), computed-tomography (CT) (d-e/inset) and echocardiography (f-m).*

Based on these promising results, in a further step, the orthotopic application of this one-step intervention concept was challenged. After development and detailed adaption of the

TEHV design to the anatomic conditions of an orthotopic aortic valve, in a subsequent trial (n=12), marrow stromal cell based tissue engineered heart valves were for the first time implanted into the orthotopic position of the high pressure system using sophisticated transcatheter delivery systems (figure 11).

In all twelve sheep of this proof-of concept trial the implantation was successful and valve functionality was confirmed using fluoroscopy and trans-esophageal echocardiography. While displaying an ideal opening and closing behaviour with a sufficient coaptation and a low pressure gradient (max 11mmHg), there were no signs of coronary occlusion or mal perfusion. In addition, mitral valve functionality was confirmed without any signs of regurgitation due to the stent expansion (Emmert et al, unpublished data).

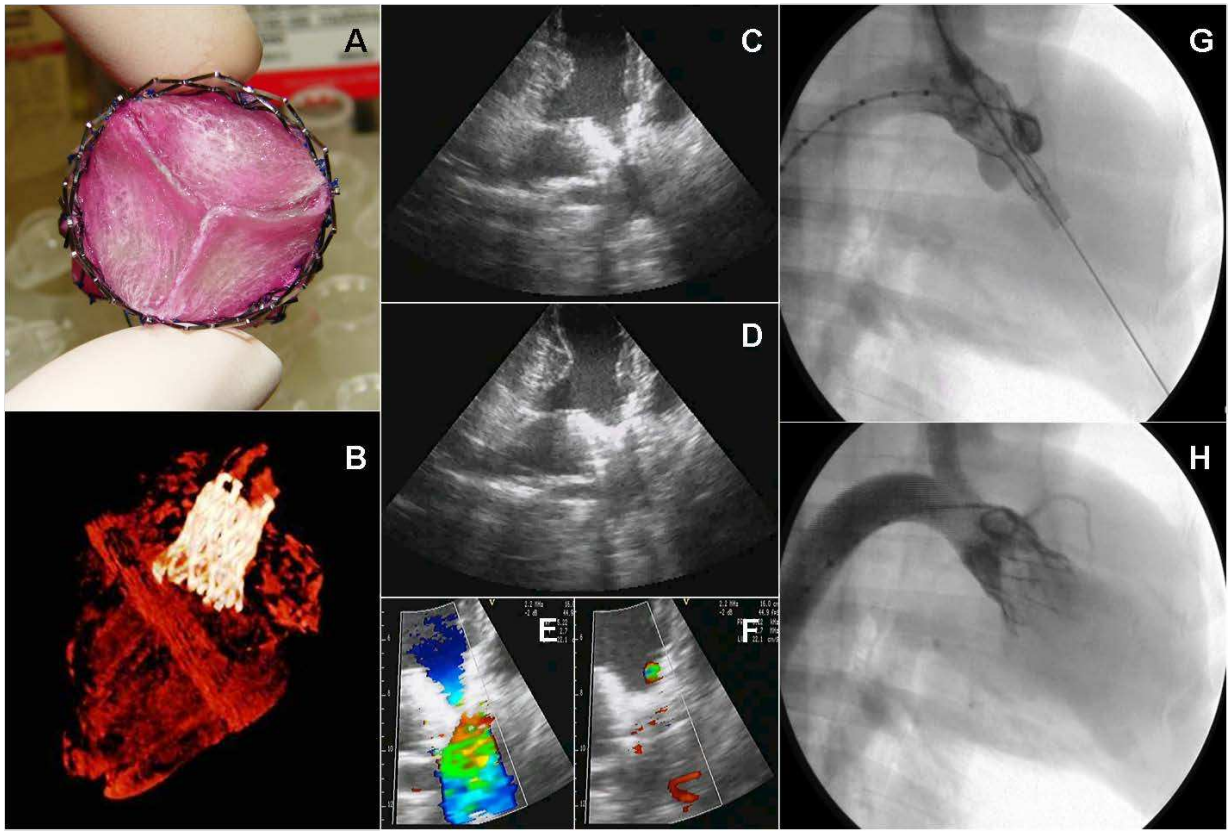

Figure 11: First orthotopic, transapical, tissue-engineered heart valve implantation into the aortic position: *Based on the one-step procedure, marrow stromal cell based tissue engineered heart valves (A) were transapically implanted into the orthotopic, aortic position. Optimal positioning was confirmed using 3D computed tomography reconstruction imagery (B) and fluoroscopy (G). In the trans oesophageal echocardiography the implanted heart valves displayed excellent functionality with sufficient opening and closing (C-F) which was also confirmed in the fluoroscopy (H). In particular, there were no signs of coronary occlusion or perfusion compromise (Emmert et al, unpublished data).*

So far, successful TEHV implantations have only been reported for the low-pressure system. These are the first reports demonstrating the feasibility of the minimal-invasive, catheter-

assisted implantation of TEHV into the systemic circulation with an adequate valvular functionality based on an easily accessible, clinically relevant cell-source (25, 34) and minimally-invasive techniques for both, cell-harvest and delivery. Its long term durability proven, such autologous and living heart valves may hold potential to overcome the limitations of the currently used bioprosthetic valves inherently prone to calcification and progressive dysfunctional degeneration.

2.5 Fetal Transapical Stent Delivery into the Pulmonary Artery: Prospects for prenatal heart valve implantation

Emmert MY*, *Weber B*, Behr L, Brokopp CE, Frauenfelder T, Kretschmar O, Falk V, Hoerstrup SP. Fetal Transapical Stent Delivery into the Pulmonary Artery: Prospects for prenatal heart valve implantation.*

*Eur J Cardiothorac Surg. 2011 Jul 7. [Epub ahead of print]; *contributed equally*

For congenital applications, the concept of autologous tissue engineered heart valves may play a crucial role providing next generation constructs due to their high capacity of growth. Importantly, the prenatal correction of congenital cardiac defects, as a surgical repair at the earliest point in time, would be desirable and may be associated with significant therapeutic advantages with regards to less intrauterine cardiac mal-development, reduction of severity of postnatal disease and enhanced (scarless) healing. Therefore, the relief of obstructive malformations, such as heart valve stenoses, using interventional procedures has been suggested to promote functional recovery of the affected ventricle (100-102).

In recent pre-clinical as well as pioneering clinical attempts the principal feasibility of fetal cardiac interventions has been reported, but has mainly focused on percutaneous and/or trans-uterine interventional approaches for treating severe aortic and pulmonary stenoses (37, 101-104), while open fetal cardiac surgery for valvotomy has not yet been successfully achieved. In 2008, Schmidt and associates (105) already demonstrated the feasibility of percutaneous ultrasound-guided cardiac stenting of the atrial septum in the ovine fetal model.

In this pilot study, the principal technical feasibility and acute safety of an intra-uterine, catheter-based transapical stent implantation into the pulmonary artery of fetal sheep was assessed using a novel fetal hybrid intervention technique which could serve as a potential future route for minimally invasive prenatal heart valve implantation procedures.

In brief, pregnant Pre-Alp sheep between 122 to 128 days' gestation underwent a midline laparotomy. The fetus was left in utero or partially externalized and its chest was opened via a left-sided mini-thoracotomy. The fetal heart was cannulated and a guide-wire was introduced through the ductus arteriosus into the aorta. A 14-French delivery system was then mounted onto the guide-wire and advanced to the defined landing zone located 3.0-5.0 mm distal to the pulmonary valve annulus, where the stent was delivered. The positioning of the stent was confirmed using echocardiography, angiography as well as computed tomography. Although the introduction of the delivery device into the right ventricle was difficult in all fetuses and significant blood loss occurred in one of the animals, the stent deployment was successful in all animals and all fetuses survived. The surgical procedure was approximately 60 minutes, lasting from maternal laparotomy to the end of post-deployment in vivo evaluation. In all animals contrast angiography displayed normal perfusion of the pulmonary vasculature as well as the ductus arteriosus and no fetal cardiac arrhythmia or bradycardia was observed during the procedures.

In one of the fetuses (F1) the stent was deployed supra-valvular into the pulmonary artery. While an initial echo analysis confirmed the stent position clearly proximal to the ductus arterious Botalli (DAB), at angiography the distal stent ending was detected to reach into the proximal part of the DAB, indicating a slight stent migration after deployment. However, as confirmed by echo and angiography this migration did not compromise the DAB or PA perfusion in this fetus. In another fetus (F2) echocardiography revealed that one of the pulmonary valvular leaflets could not be fully identified suggesting some impairment of the cusp by the proximal stents struts. In the third fetus (F3) no signs of migration or pulmonary cusp impairment were found in vivo indicating optimal positioning.

After in vivo assessment, fetuses were harvested and examined with regard to stent position, native leaflet impairment or further procedural complications. In all fetuses post mortem analysis revealed strut impregnations on the wall of the pulmonary artery, but lacking any laceration or perforation of the vessel wall. In one fetus (F2) the intraoperative sonographic finding of a too proximal stent positioning could be confirmed as one of the native pulmonary

leaflets was partly impaired by one of the proximal stent strut endings. Also in fetus F1 the angiographic stent position could be confirmed with the distal stent ending reaching into in the proximal DAB. In fetus F3 the stent was situated in the distal pulmonary artery not covering any of the native pulmonary leaflets. No signs for thoracic bleeding or stent-associated obstruction were observed.

The presented results show the principal technical feasibility of a fetal stent implantation via a catheter-based technique using the transapical route. Even if percutaneous or fetoscopic transapical cardiac approaches would be desirable, they lack control of direct fetal cardiac hemorrhage – a major issue in fetal cardiac catheterization. The presented trans-uterine open-chest closed-heart hybrid technique represents a possible alternative for future approaches, also allowing for the insertion of large devices, such as heart valve delivery systems. Further long-term studies are necessary to evaluate fetal myocardial tolerance and long-term survival of the presented technique before it may be applied to the clinical setting and could serve as a potential future route for minimally invasive prenatal heart valve implantation procedures.

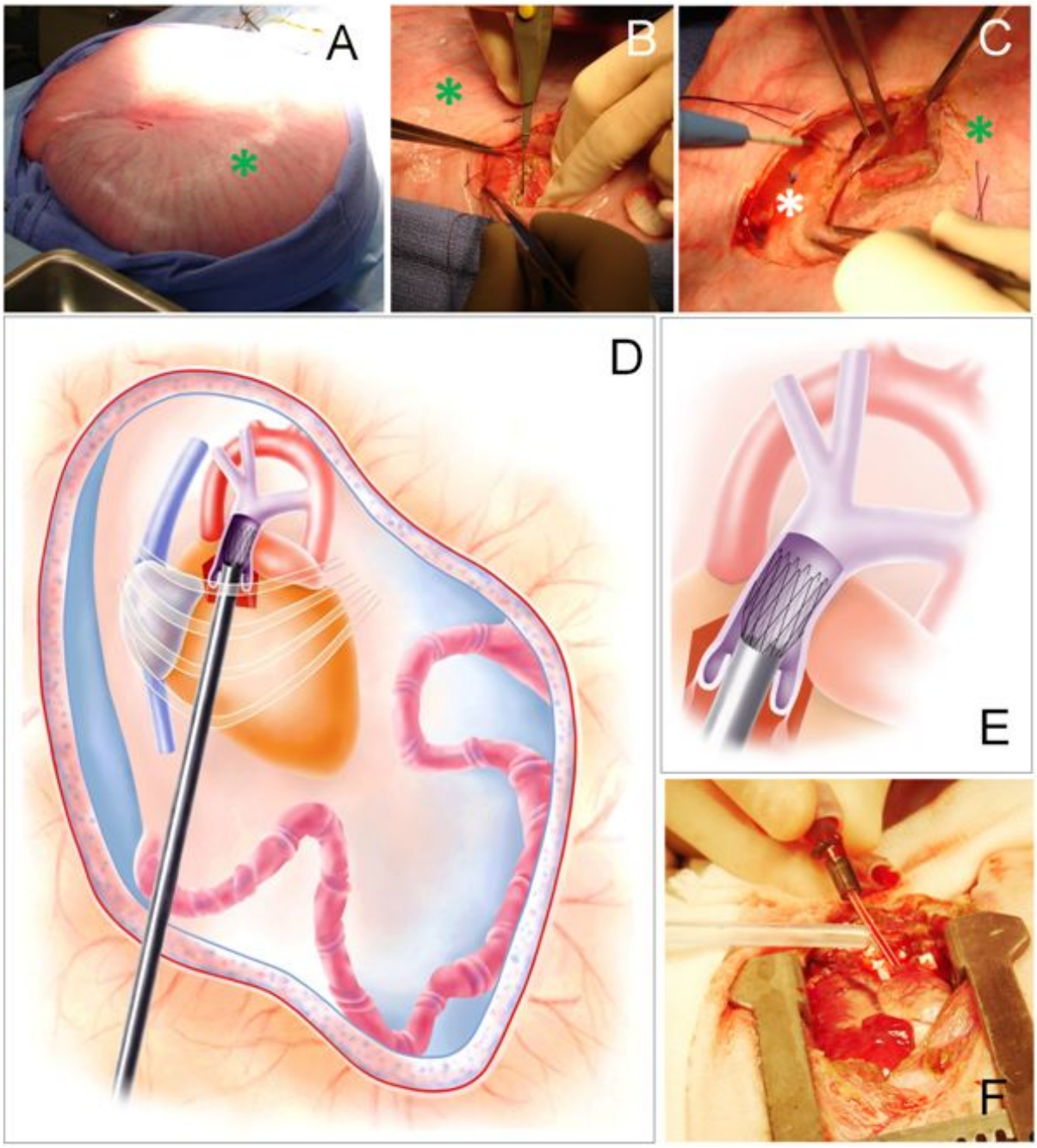

Figure 12: The fetal open-chest in-utero transapical hybrid technique. *After exteriorization of the uterus (A), a small incision was made in the uterine wall (B, *A-C: uterus), and the festal chest was opened (C). The 16 Gauge catheter was inserted into the apex of the right ventricle (F) and the delivery system is advanced to the landing zone. The stent is then deployed supra-valvular into the distal portion of the pulmonary artery (D-F).*

3. Summary of Habilitation Thesis

Valvular heart disease represents a major cause of morbidity and mortality worldwide. Minimally-invasive valve implantation techniques are rapidly evolving as alternative treatment option for the management of patients with valvular heart disease. However, despite these technical advances, the currently available valvular prostheses for valve replacement procedures are bio-prosthetic and as such inherently prone to calcification and progressive dysfunctional degeneration. Tissue engineering technologies aiming at the fabrication of cell based living heart valves have created substantial clinical hope and expectations to overcome these limitations. Particularly for pediatric applications, the growth potential of tissue engineered heart valves addresses an unmet medical need and may broaden the future clinical application of trans-catheter valves beyond elderly high-risk patients. Furthermore the combination with the specific use of stem cells may further increase the regenerative potential of tissue-engineered constructs. In particular, marrow stromal derived stem cells have been repeatedly suggested to represent an ideal cell source for cardiovascular tissue engineering. A clinically relevant heart valve tissue engineering concept would ideally comprise both minimally-invasive techniques for cell harvest and valve implantation.

The articles summarized here, demonstrate the systematic development of clinically relevant tissue engineered heart valve concepts by combining the use of stem cells, *state-of-the-art* tissue engineering protocols and minimally invasive valve implantation techniques.

In the article *`Minimally-Invasive Implantation of Living Tissue Engineered Heart Valves - A Comprehensive Approach from Autologous Vascular Cells to Stem Cells` (Schmidt, …Emmert et al., 2010)* for the first time, the principal feasibility of merging tissue engineering and minimally-invasive valve replacement technologies using adult stem cells was successfully demonstrated. Tri-leaflet heart valves fabricated from biodegradable synthetic scaffolds and autologous vascular or stem cells were integrated in self-expanding stents and

minimally-invasively implanted as pulmonary valve replacements in sheep. In vivo functionality was confirmed for up to eight weeks and histology revealed layered neo-tissues with endothelialized surfaces after sacrifice.

As a meticulous understanding of the underlying mechanisms of neo-tissue formation and matrix remodeling is crucial with regards to future clinical applications of tissue engineering technologies, matrix metalloproteinases (MMP) were identified to play a key role of matrix remodeling, tissue homeostasis and functional growth. Based on a previous study elucidating the concept of growth (Hoerstrup et al, 2006), MMPs were demonstrated to contribute to increased matrix remodeling, native analogous tissue formation and functional growth in tissue-engineered vascular grafts (TEVGs) in vivo in a growing lamb model *(`Tissue engineered vascular graft remodeling in a growing lamb model: expression of matrix metalloproteinases` - Cummings, …Emmert et al, 2011).*

In a further step, the feasibility of generating injectable, marrow stromal cell-based, autologous, living tissue engineered heart valves (TEHVs) and implantation in a one-step intervention in non-human primates was investigated *(`Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates` - Emmert*, Weber* et al, 2011; *contributed equally)*. Within the same intervention, autologous bone marrow-derived mononuclear cells were harvested, seeded on biodegradable scaffolds, integrated into self-expanding nitinol stents and transapically implanted as pulmonary valve replacements into non-human primates. For the first time, the feasibility of heart valve tissue engineering based on a minimally invasive technique for both cell harvest and valve delivery in a one-step intervention could be demonstrated while avoiding any need of an in vitro bio-reactor phase.

Based on this novel approach and in order to broaden the future clinical application of trans-catheter tissue engineered heart valves, in a subsequent step this concept was also successfully applied to the high-pressure system of the systemic circulation *(`Transapical Aortic Implantation of Autologous Marrow Stromal Cell-based Tissue Engineered Heart*

Valves – First Experiences in the Systemic Circulation` - Emmert, Weber et al, 2011). The fabricated TEHVs were transapically delivered into the descending aorta (distal to the brachiocephalic-trunk) and brachiocephalic-trunk of sheep. To achieve a sufficient loading, native valve incompetence was created by applying the *Hufnagel* procedure prior to implantation. Post mortem analysis displayed fully intact TEHVs and in particular well defined leaflets showing coaptation, while there were no signs of pressure damage, leaflet rupture or thrombus formation detectable.

In the context of pediatric applications, TEHVs appear to be particularly beneficial due to their high capacity of growth. Importantly, the prenatal correction of congenital cardiac defects, as a surgical repair at the earliest time point, would be desirable and may be associated with significant therapeutic advantages with regards to less intrauterine cardiac mal-development, reduction of severity of postnatal disease and enhanced (scarless) healing. Therefore, as a next step in the frame of a pilot study, the principal technical feasibility and acute safety of intra-uterine, fetal stent implantation into the pulmonary artery via a catheter-based transapical hybrid technique in an ovine fetal model was demonstrated, being the first step towards possible future minimally invasive prenatal heart valve implantation procedures *(`Fetal Transapical Stent Delivery into the Pulmonary Artery: Prospects for prenatal heart valve implantation` - Emmert*, Weber* et al, 2011; *contributed equally).*

The results of this habilitation thesis demonstrate that the combination stem-cell based heart valve tissue engineering and minimally invasive implantation techniques represents a promising, clinically relevant approach which may be particularly beneficial for paediatric patients due to its high capacity of growth. Its long-term durability proven, such autologous and living heart valves with repair properties may represent the next generation of transcatheter heart valves overcoming the limitations of the currently used prostheses.

4. Conclusions & Outlook

More than 50 years ago, Dr. D.E. Harken, a pioneer in the field of heart valve surgery, was the first who successfully implanted a heart valve into the subcoronary, aortic position in a patient suffering from severe aortic stenosis (106). After this milestone achievement representing a breakthrough in the field of heart valve diseases, the evolution of heart valve prostheses has undergone rapid changes and made significant advances over the past decades (figure 13). While the initial valve prostheses were fabricated from mechanical components based on concept models derived from the aviation industry, the field has changed stepwise into the direction of biological prostheses over the past years. In parallel, even though the conventional surgical technique of heart valve replacement has served as a very safe and reliable method for many years, the valve replacement techniques have continuously changed into the direction of minimally invasive, transcatheter-based approaches (figure 13).

Despite these tremendous advances over the past fifty years with regards to the refinement of heart valve prostheses as well as the surgical technologies for implantation, major issues have remained unsolved. Although current available prostheses demonstrate excellent structural durability (9, 12-14), the currently available bio-prostheses for minimally invasive, transcatheter-based procedures are still inherently prone to progressive degeneration and calcification requiring redo surgery within 10-15 years after implantation and therefore suggesting their clinical indication primarily in the elderly, high-risk patients (7). Furthermore, the lack of growth capacity as well as in vivo repair and remodeling properties remain key issues with particular regards to congenital applications.

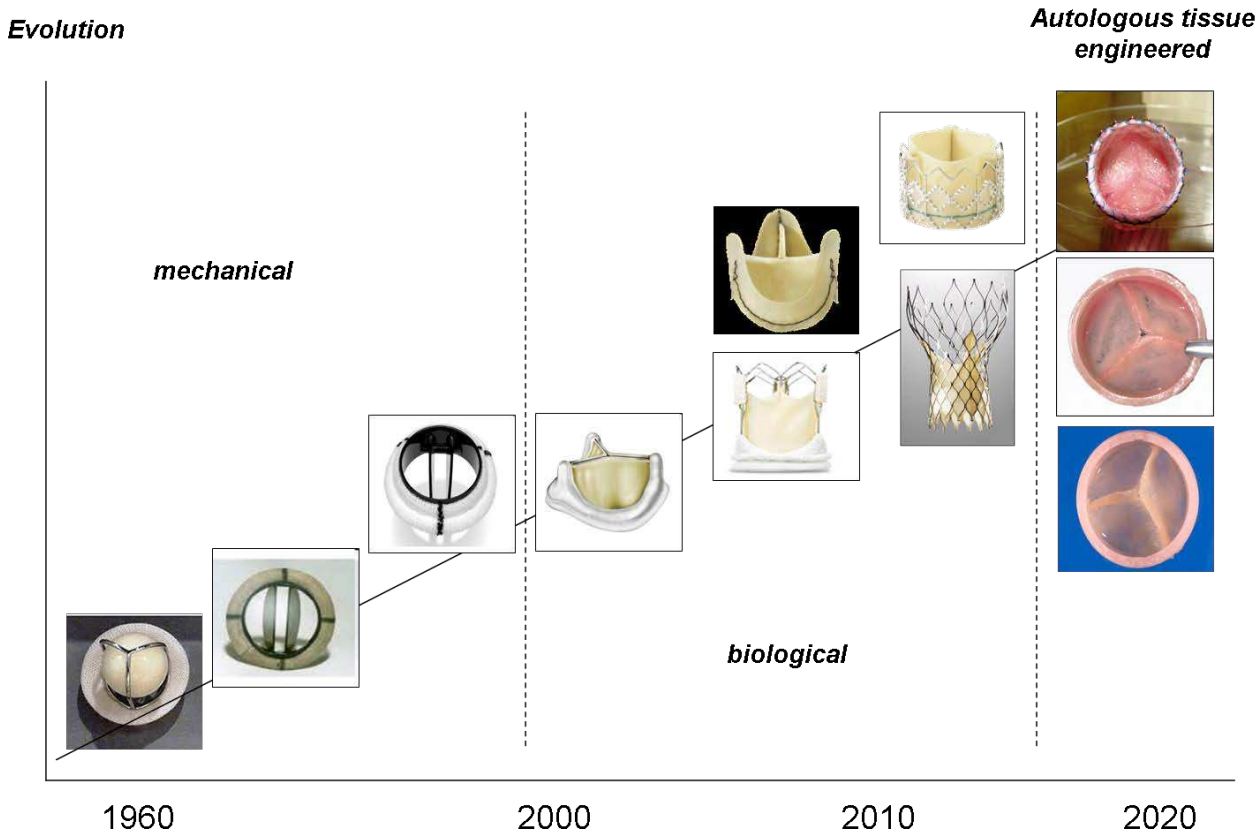

Figure 13: The Evolution of Heart Valve Prostheses: *After Dr. D.E. Harken was the first who successfully implanted a heart valve into the subcoronary, aortic position in a patient suffering from severe aortic stenosis in 1960, the evolution heart valve prostheses has undergone rapid changes and made significant advances over the past decades. In parallel, also the valve replacement techniques have continuously changed into the direction of minimally invasive, transcatheter-based approaches. The concept of heart valve tissue engineering offering the potential of growth and remodeling, may represent an ideal, next generation therapy concept for trans-catheter heart valve replacements (Emmert & Hoerstrup; unpublished concept figure)*

Despite the rapid evolution in the treatment of valvular diseases, these limitations clearly indicate that current therapy concepts of heart valve replacements are still suboptimal and the ideal approach needs to be developed (11). Interestingly, it was also Dr. D.E. Harken, who already defined in the 1950th the essential characteristics of the ideal valve prosthesis. In his so-called `Ten Commandments` (107), the pioneer Harken summarized the major aspects of the ideal heart valve construct, including durability, absence of thrombogenicity, resistance to infections, lack of antigenicity, and the potential of growth. He stated the key characteristics of natural, living, heart valves (107).

To overcome these limitations, the concept of heart valve tissue engineering has been suggested as a promising approach and has created substantial hope as well as expectations. The major goal of tissue engineering concept is to fabricate living, autologous

heart valves exactly comprising the properties of their native counterpart. The idea of this tissue engineering heart valve concept which is not associated with any immune reaction, thromboembolic events or degeneration processes, while offering the potential of growth and remodeling, may represent the ideal next generation therapy concept for heart valve replacements.

In this habilitation thesis, the principal feasibility and safety of heart valve tissue engineering concepts in a translational, preclinical setting is systematically demonstrated. The data presented provide systematic evidence of the development of clinically relevant tissue engineered heart valve concepts by combining the use of stem cells, *state-of-the-art* tissue engineering protocols and minimally invasive valve implantation techniques. These strategies may represent a promising, future approach being particularly beneficial for paediatric patients due to its high capacity of growth addressing an unmet medical and may prevent children with congenital malformations from high risk redo operations. Furthermore, the implementation of these concepts into the clinical routine may also have a vast impact with regards to economic as well as social aspects. In both scenarios the congenital as well as adult setting, the application of autologous, living heart valves may prevent the patients from the necessity of reoperations saving a lot of financial and logistical resources. Subsequently, the introduced concept of using endogenous stem cell potential of a patient in a one-step intervention, while avoiding any need for an expensive and logistically demanding in vitro bio-reactor phase, may represent an ideal and highly efficient concept especially for patients in the developing countries with limited financial and technical resources.

However, despite of its huge potential to significantly improve the therapy of valvular heart disease and despite all enthusiasm, several key steps have to be completed before the standardized implementation of heart valve tissue engineering into the clinical arena will be possible. Besides the principal demonstration of the long-term safety and efficacy of an implanted construct to prevent from catastrophic failure, pre-clinical guidelines defining how to characterize the safety, efficacy, and quality of a tissue engineered construct need to be established (108). In brief, future research directions (summarized in table 2) should focus on

the fundamental understanding of underlying remodeling and repair mechanisms including a better knowledge of the biochemical and immunological characteristics of the cells and tissues undergoing in vitro growth. In addition, the disposition of implanted structures to typical biomaterial tissue interactions of medical devices, such as calcification, thrombosis and excessive inflammatory response have to be elucidated (109).

Another key consideration is the important aspect that currently available valve prostheses reveal a certain, predictable behaviour with regards to durability and biocompatibility; while tissue engineered constructs, potentially relying on in vivo remodeling, may show substantial variability among different patients due to the heterogeneity of the physiological tissue remodeling potential. For this reason, the development of sufficient clinical guidelines defining the inclusion criteria for specific patient cohorts with regards to safety, efficacy and quality of the engineered constructs is mandatory (109). Therefore, the identification of biomarkers as independent predictors as well as conventional or innovative imaging modalities may represent useful and important tools to assess the success, but also the failure of an implanted construct.

From the logistical point of view when considering initial clinical trials (pilot study), laboratory processes and logistics need to be adapted in according to the good manufacturing practice (GMP) guidelines providing a maximum degree of process control and bio-safety. Ideally, in the context of a classical tissue engineering approach, an exemplary algorithm would comprise the following steps: (a) isolation of autologous cells from designated sources (i.e. by a bone marrow puncture or adipose tissue aspiration under local anesthesia), (b) differentiation and expansion of cells and engineering of a heart valve prostheses in vitro; and (c) following defined quality criteria (biological, histological, bio-safety), re-implantation of living autologous valve replacements into patients after a defined time period of a maximum of six to eight weeks (109).

Challenges	Research Directions
TEHV components and their interactions are complex, heterogeneous and dynamic	Define cell/scaffold/bioreactor combinations that optimize construct composition and properties (in vitro)
Correlation of in vitro generated construct structure and properties with in vivo outcomes has not been demonstrated	Determine and validate correlations between in vitro conditions, elements, structure, properties and in vivo function
Quality control of construct structure and function is likely to be difficult	Develop guidelines, tools and metrics for the pre-implantation characterization of TEHV structure, function and quality
Animal models may not reliably predict human outcomes	Develop and validate animal models that will test key biological processes and correlate with human outcomes
TEHV structure is likely to be evolving in vivo and ongoing function may be less predictable than with conventional valve replacement technology	Develop guidelines, tools and metrics for the in vivo characterization of dynamic TEHV structure, function and quality
TEHV function will depend upon patient response to implantation and integration with the recipient's tissues more than with conventional valve replacement, and individual patient responses may be highly variable	Identify/validate biomarkers both predictive of and assess patient variability in implant success/failure and capable of non-invasive in vivo monitoring and potential control
Remodeling processes after implantation may release or change seeded cells and recruit host cells	Develop tools to monitor the fate of transplanted and endogenous cells (location, function, viability, phenotype)
Regulatory processes and approaches are not yet well established	Create suitable regulatory approaches to engineered tissue valves that will ensure safety and efficacy

Table 2: Key steps and future research challenges for the clinical translation of Heart Valve Tissue Engineering *(adapted from Schoen, Review; Current Opinion in Biotechnology, 2011); TEHV = Tissue Engineered Heart Valve*

In conclusion, the data presented in this habilitation thesis systematically demonstrate that the combination of sophisticated stem cell approaches, *state-of-the-art* tissue engineering protocols and minimally invasive valve implantation techniques represents a promising, clinically relevant approach which may be particularly beneficial for paediatric patients. Its long-term feasibility proven and considering the above mentioned future research challenges, the concept of autologous, living heart valves may represent the next generation of trans-catheter heart valves overcoming the limitations of the currently used prostheses and may be rapidly translated into a clinical setting.

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6. Appendix